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NOTES

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Targeting Urokinase-Type Plasminogen Activator Receptor on Human Glioblastoma Tumors With Diphtheria Toxin Fusion Protein DTAT

Daniel A. Vallera, Chunbin Li, Ni Jin, Angela Panoskaltsis-Mortari, Walter A. Hall

Background: **The prognosis for patients with brain cancer is poor, and new therapies are urgently needed. Recombinant toxic proteins that specifically target tumor cells appear to be promising. Urokinase-type plasminogen activator (uPA) receptor (uPAR) is expressed on the surface of glioblastoma and some other tumor cells and endothelial cells. We synthesized a recombinant fusion protein, DTAT, which contains the catalytic portion of diphtheria toxin (DT) for cell killing fused to the noninternalizing amino-terminal (AT) fragment of uPA, and investigated its effectiveness in targeting uPAR-positive tumor cells.** ***Methods: In vitro*** **cytotoxicity f DTAT was measured by cell proliferation assays. For** ***in vivo*** **studies, athymic nude mice (four to five animals/group) bearing uPAR-expressing human glioblastoma (U118MG) cell-induced tumors were injected with DTAT or control pr tein. Tumor volume was assessed over time, and differences between treatments were analyzed by Student's *t* test. Effects of DTAT on body organ systems were evaluated in normal, tumor-free C57BL/6 mice histologically and functionally by serum enzyme tests. All statistical tests were two-sided.** ***Results: In vitro,*** **DTAT was highly potent and selective in killing uPAR-expressing glioblastoma cells (U118MG, U373MG, and U87MG) and human umbilical vein endothelial cells.** ***In vivo,*** **compared with mice treated with control proteins, DTAT caused a statistically significant ($P = .05$) regression of small U118MG cell-induced tumors in all mice. Control fusion proteins that did not react with glioblastoma cells had no effect on tumor growth. DTAT given to tumor-free C57BL/6 mice had little effect on kidney, liver, heart, lung, and spleen histologies. Serum analysis in the same mice showed no elevation in blood urea nitrogen, indicating lack f effect on kidney function but a statistically significant ($P = .046$), albeit non-life-threatening, elevation in liver alanine aminotransferase levels.** ***Conclusion:*** **DTAT may have potential f r intracranial glioblastoma therapy because of its ability to target tum r cells and tumor vasculature simultaneously and its apparent lack of systemic effects. [J Natl Cancer Inst 2002;94:597–606]**

Among adolescents and adults in the age range of 15–34 years, central nervous system malignancies are the third leading cause of cancer-related death *(1)*. Moreover, the 2-year survival rate of patients is extremely low at less than 20% *(2)*. Therapeutic approaches have been complicated by the fact that there has been no tumor-specific marker that could be targeted in the majority of patients *(3)*. The discovery that certain receptors are selectively overexpressed on glioblastoma multiforme (GBM), an aggressive form of brain cancer, has raised new interest in targeting this cancer with fusion proteins (FPs). These FPs are experimental therapeutic agents consisting of a targeting ligand coupled to a potent toxin *(4)*. Although attractive in concept, these FPs have had limited clinical promise because of their failure to concentrate at the site of tumor *(5)*. Unfavorable tumor vasculature dynamics (high interstitial pressures) and distance needed to be traveled by the injected protein result in its inefficient distribution to target tissue *(6)*. Injection of FP directly into the tumor may circumvent these problems because therapy can be concentrated at the site of the tumor, thereby diminishing the risk to nontarget organs. Brain tumor anatomy lends itself to intratumoral therapy, and clinical response to intratumoral FP therapy for brain cancer has far exceeded that of other cancer types—in excess of 50% *(7)*.

Investigators have studied interleukin 13 (IL-13) as an FP ligand and have shown that although normal cells may express the receptor, they remain poorly responsive to IL-13-FP, probably because of limited receptor expression and different receptor structures *(8)*. IL-13-FP made with *Pseudomonas* exotoxin is

Affiliations of authors: D. A. Vallera, C. Li, N. Jin, Section on Molecular Cancer Therapeutics, Department of Therapeutic Radiology–Radiation Oncology, University of Minnesota Cancer Center, Minneapolis; A. Panoskaltsis-Mortari (Department of Pediatrics) and W. A. Hall (Department of Neurosurgery), University of Minnesota.

Correspondence to: D. A. Vallera, Ph.D., University of Minnesota Cancer Center, Mayo Mail Code 367, Harvard St. at East River Rd., Minneapolis, MN 55455 (e-mail: valle001@tc.umn.edu).

See "Notes" following "References."

specifically cytotoxic *(9)*. Recently, we showed that diphtheria toxin (DT)-IL-13 fusion toxin was effective against gliomas that express high levels of IL-13 receptor (IL-13R) *(10)*. Despite its well-documented potency and selectivity, use of IL-13-FP is limited because all glioblastomas do not express IL-13R. Data in this paper, including nude mice studies, indicate that certain GBM tumors are unaffected by therapy with IL-13-FP. Therefore, based on an extensive body of literature supporting the expression of urokinase-type plasminogen activator (uPA) receptor (uPAR) on glioblastoma [reviewed in *(11)*], we devised an alternative strategy for DT-IL-13-unresponsive tumors. The uPAR was targeted because, in addition to its expression on tumor cells *(12–16)*, it is also expressed on endothelial cells [reviewed in *(17)*]. Endothelial cell expression of uPAR is an advantage because other investigators have successfully targeted tumor neovasculature with FP, thus demonstrating that destruction of the tumor microvasculature inhibits tumor growth *(18)*.

To simplify our approach, we cloned a hybrid molecule by using the amino-terminal (AT) fragment (ATF) of uPA. Urokinase is sequestered at the cancer cell surface by its receptor uPAR, thereby activating circulating plasminogen protease [reviewed in *(19)*]. This enhances proteolysis of extracellular adhesion molecules such as collagen and fibronectin, thus promoting tumor invasion. We chose the ATF domain because it includes the molecular binding region and a fusion protein made with ATF and saporin-inhibited human uPAR-expressing cells *(20)*. The ATF domain completely lacks the catalytic domain of uPA but possesses an endothelial growth factor (EGF)-like or growth factor domain that comprises the receptor binding sequence of human uPA *(21)*. Upstream of the ATF, DT's catalytic region was attached to render the molecule cytotoxic. A single DT molecule in the cytosol can kill a cell *(22)*. The translocation-enhancing region of DT was included in the design to enhance the toxicity of the FP. To test the idea that an agent with the ability to bind to both tumor and tumor vasculature would have potent anti-glioblastoma effects, we synthesized and tested *in vivo* and *in vitro* the effectiveness of DTAT, which can target uPAR on the cell surface. We hypothesize that the DTAT molecule will be highly active against glioblastoma cells.

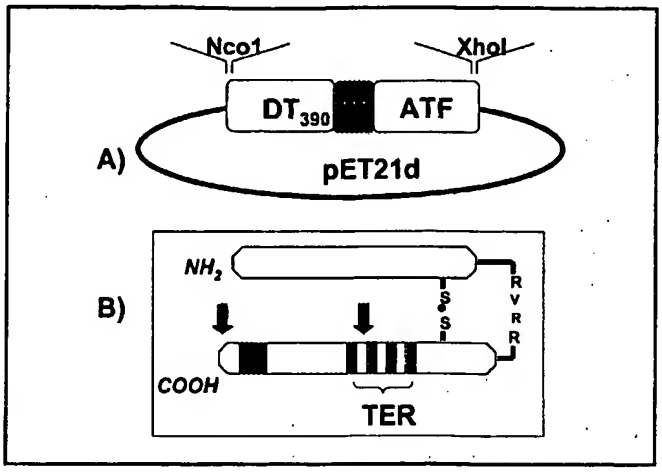

Fig. 1. A) Construct encoding the DTAT (diphtheria toxin [DT]-amino terminal [AT] fragment of urokinase-type plasminogen activator [uPA]) gene fragment used in these studies. An NcoI/XhoI gene fragment was cloned by polymerase chain reaction and splice overlap extension encoding DT_{390}, an EASGGPE linker (black box), and the downstream 135-amino-acid AT fragment (ATF) from uPA. The gene was cloned into the pET21d expression vector forming the plasmid pDTAT.pET21d. **B)** Cartoon depicts DT_{390}, the 193-amino-acid N-terminal A chain, the 342-amino-acid B chain, a disulfide bond between the two chains, and the native binding region that has been genetically removed (145 amino acids). The region between the two black arrows depicts the native binding region. The four bands on the B chain depict the hydrophobic translocation enhancing region (TER). The RVRR is a protease-sensitive site that must be nicked in low pH endosomes to activate the toxic protein.

Materials and Methods

Recombinant DTAT

Recombinant DTAT was synthesized by a technique previously described *(23)*. The hybrid gene was constructed by the method of gene splicing (Fig. 1, A and B). An *NcoI*/*XhoI* gene fragment was cloned by polymerase chain reaction (with a splice overlap extension encoding the 390-amino-acid portion of DT [DT_{390}], an EASGGPE linker [black box in Fig. 1, panel A], and the downstream 135-amino-acid ATF from uPA) and ligated into the pET21d expression vector forming plasmid pDTAT.pET21 (Novagen, Madison, WI). The DT_{390} consists of a 193-amino-acid N-terminal A chain, the 342-amino-acid B chain, a disulfide bond between chains A and B, and the native binding region (region between the two black arrows in Fig. 1, panel B) that has been genetically removed (145 amino acids). The B chain has a hydrophobic translocation enhancing region (TER) (shown by four black bands in Fig. 1, panel B). The RVRR is a protease-sensitive site that must be nicked in low pH endosomes to activate the toxic protein. Restriction endonuclease digestion and DNA sequencing analysis (University of Minnesota Microchemical Facility) were used to verify that the hybrid gene had been cloned in frame. Plasmid was transformed into the *Escherichia coli* strain BL21(DE3) (Novagen). Expression was induced and protein was refolded and purified from inclusion bodies. The pellets were washed three times with Triton X-100 buffer and four times with wash buffer (50 m*M* Tris, 50 m*M* NaCl, and 5 m*M* EDTA buffer by briefly homogenizing with a tissuemizer and incubating for 5–10 minutes. Inclusion bodies were collected by centrifugation at 24 000*g* for 50 minutes. Solubilization of the inclusion body pellet was achieved by sonicating in denaturant buffer consisting of 7 *M* guanidine, 0.1 *M* Tris (pH 8.0), and 2 m*M* EDTA. To remove insoluble material, the solution was centrifuged at 40 000*g* for 10 minutes, and the supernatant was collected. Renaturation was initiated by a rapid 100-fold dilution of the denatured protein into chilled refolding buffer consisting of 0.1 *M* Tris (pH 8.0), 0.5 *M* L-arginine, and 2 m*M* EDTA. The samples were incubated at 10 °C for 48 hours. Ultrafiltration was performed against 20 m*M* Tris (pH 7.8) with a spiral membrane ultrafiltration cartridge on Amicon's CH2 system (Amicon, Beverly, MA). Samples were loaded on a Q-Sepharose (Sigma-Aldrich, St. Louis, MO) ion exchange column and eluted with 1 *M* NaCl in 20 m*M* Tris (pH 7.8). The protein was diluted fivefold and subsequently applied to a Resource Q column (Pharmacia Biotech, Uppsala, Sweden) and eluted with a linear salt gradient from 0 to 1 *M* NaCl in 20 m*M* Tris (pH 7.8) and dialyzed against 1× phosphate-buffered saline (PBS). Ion exchange chromatography conditions were based on the estimated isoelectric point of 6.1 derived with the use of ISOELECTRIC (Wisconsin Package version 10.0-UNIX; Genetics Computer Group, Madison, WI). The main peak from the Resource Q column was further purified by size-exclusion chromatography on a TSK 250 column (TosoHass, Montgom-

eryville, PA). Standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis was performed to assess purity. Preparations of control DT_{390} containing FPs with either mouse or human interleukins (e.g., DT-m-IL-4, DT-h-IL-2, DT-h-IL-13, and DT-m-IL-3) have been previously reported *(23–25)*.

Cell Lines and Antibodies

The cell lines U118MG, U87MG, U373MG, and T98G (with suffix G) were derived from human patients diagnosed with GBM and were obtained from American Type Culture Collection (Manassas, VA). Neuro-2a (a murine neuroblastoma cell line), Daudi (derived from human Burkitt's lymphoma), and SKBR3 (a human mammary gland adenocarcinoma line) were also obtained from ATCC. They were all maintained in RPMI-1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; BioWhittaker), 2 m*M* L-glutamine (Life Technologies, Rockville, MD), 0.1 m*M* nonessential amino acids (Life Technologies), 1.0 m*M* sodium pyruvate, 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Technologies). Human umbilical vein endothelial cells (HUVECs), used within seven passages, were obtained from Dr. S. Ramakrishnan (University of Minnesota) and were maintained in Medium 199 (Life Technologies) containing 15% heat-inactivated FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1 mL epidermal cell growth media (BioWhittaker). All the cells were maintained at 37 °C in a humidified incubator with 5% CO_2/95% air and were passaged two to three times per week.

Neutralization experiments were performed by using polyclonal rabbit anti-human urokinase immunoglobulin G (IgG) as well as a murine IgG2a monoclonal antibody against human uPAR obtained from American Diagnostica (Greenwich, CT). Anti-IL-4 antibody (rat anti-mouse IgG1 from clone 11B11) *(26)* was used as a control for the blocking experiments (described in Fig. 4, B). To facilitate human tumor growth in nude mice, rabbit anti-asialoGM1 (Wako Chemicals USA, Richmond, VA) was administered.

Proliferation Assay

The *in vitro* cytotoxicity of DTAT and other FPs was measured by inhibition of DNA synthesis *(27)*. Cells at 10^4/well were plated in 100 μL of culture medium in a 96-well flat-bottomed tissue culture plate and incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Various concentrations of DTAT diluted in culture medium were added in 100-μL volumes and incubated for 48 or 72 hours. One μCi of [methyl-^{3}H]thymidine (Amersham Pharmacia Biotech, Little Chalfont, U.K.) was added at the beginning of the last 8 hours of incubation. The cells were washed and harvested on glass fiber filters, and the incorporation of radioactivity was quantified. All cytotoxicity assays were performed in triplicate and expressed as a percentage of control response, i.e., incorporation of [^{3}H]thymidine in cells incubated without toxin. The assays were repeated at least two to four times, showing that the results were highly reproducible. The concentration of DTAT at which 50% inhibition of DNA synthesis (IC_{50}) occurred was calculated. For blocking experiments, antibodies were pre-incubated with the toxins for 30 minutes at 37 °C, and the experiment was performed as described above.

In Vivo Mouse Tumor Studies

Six- to eight-week-old female athymic nu/nu nude mice (for tumor studies) and C57BL/6 mice (for toxicity studies) were purchased from the National Institutes of Health (Bethesda, MD) and maintained in microisolator cages under specific pathogen-free conditions as set forth by the Department of Research Animal Resources (University of Minnesota, Minneapolis). On day 2 and day 4, 25 μL of anti-asialoGM1 diluted in 175 μL PBS was injected intraperitoneally into each nude mouse. Anti-asialoGM1 is an immunosuppressive agent that reacts with mouse natural killer (NK) cells, mouse monocytes, and fetal thymocytes, thereby enhancing tumor growth. On day 0, U118MG cells (6×10^6/0.1 mL culture medium) were injected subcutaneously into the right flank of each mouse. Each treatment group consisted of four or five animals. Mice were examined every 2–3 days. Palpable tumors with tumor volume larger than 0.15 cm^3 were treated by intratumoral injection. Twenty micrograms of DTAT diluted in 50 μL of PBS was injected every other day. Five such treatments were given to each mouse. DTAT was injected from three different directions by using a 0.5-cc insulin syringe with a 28-gauge needle over a period of approximately 1 minute. Control mice received an irrelevant FP DT-h-IL-2 (FP containing DT and human IL-2) or PBS injections. Tumor size was measured by caliper every 2–3 days. The approximate tumor volume was calculated as a product of length, width, and height.

Histology

A group of normal C57BL/6 mice without tumors were treated in the same manner as in the nude mouse experiment. At the end of the treatment, the mice were sacrificed and autopsied and their tissues were taken for histopathologic analysis as described *(28)*. All samples were imbedded in OCT compound (Miles Laboratories, Elkhart, IN), snap frozen in liquid nitrogen, and stored at –80 °C until sectioned. Four serial sections (4 μm thick) were cut, thaw-mounted onto glass slides, and fixed for 5 minutes in acetone. Slides were stained with hematoxylin and eosin (H & E) for histopathologic assessment.

Blood Urea Nitrogen (BUN) and Alanine Aminotransferase (ALT) Assays

Both assays were performed as previously described *(29)* on Kodak EKTACHEM clinical chemistry slides (Eastman Kodak, Rochester, NY) on a Kodak EKTACHEM 950 by the Fairview University Medical Center–University Campus (Minneapolis, MN). C57BL/6 mice were randomly grouped ($n = 5$) and injected with DTAT, PBS (no FP), or a control DTanti-CD3sFv fusion toxin. DTanti-CD3sFv has been previously shown to cause organ toxicity. On the day immediately following the last injection, mice were sacrificed and bled. Individual serum samples collected by bleeding the heart were studied for BUN and ALT. Minimum specimen volume was 11 μL for each assay. The BUN assay was read spectrophotometrically at 670 nm. In the ALT assay, the oxidation of NADH was used to measure ALT activity at 340 nm.

Statistical Analysis and Reproducibility

Groupwise comparisons of continuous data were made by Student's *t* test. *In vitro* experiments were repeated at least twice,

and *in vivo* experiments were repeated at least once. All statistical tests were two-sided.

RESULTS

Purity of DTAT

To assess the purity of fractions containing DTAT collected from our chromatography procedure, SDS–PAGE analysis was performed. SDS–PAGE analysis indicated that the purification schema resulted in a purity exceeding 95% for DTAT and the isoelectric point of DTAT was estimated to be 6.1. Control FPs, including DT-IL-13, were prepared and tested for purity in a similar manner.

DT-IL-13 and Killing of Glioblastoma Cell Lines

To determine whether DT-IL-13 would kill human glioblastoma cells, DT-IL-13 was tested in a tritiated thymidine incorporation assay. A different report from our group *(10)* has previously addressed the high selectivity and potency of DT-IL-13. Although DT-IL-13 in picomolar concentrations killed some glioblastoma cell lines (Fig. 2, A and B), others were not affected. Fig. 2, B, shows that DT-IL-13 inhibited the U373MG glioblastoma line (IC_{50}<0.01 nanomoles). In contrast, another glioblastoma cell line, U87MG, was inhibited only 45% when treated with a 1000-fold higher (10 n*M*) concentration of the agent. A third glioblastoma cell line, T98G, was not inhibited at all. These findings appear to be related to the IL-13R expression of U373MG (16400 binding sites/cell) and T98G (549 binding sites/cell) which have been previously reported *(30);* expression of IL-13 receptors in the U87MG cell line is not known. In other words, higher receptor numbers are associated with greater killing levels.

Comparison of Cytotoxicities of DTAT and DT-IL-13

Because uPAR is overexpressed on certain cancer cells, it was important to determine the ability of DTAT to kill glioblastoma and to discover whether it could kill certain glioblastomas that were not eliminated by DT-IL-13. In our hands, U118MG cells were not inhibited well by DT-IL-13 treatment. Fig. 2, A, shows that despite treatment with 10 n*M* DT-IL-13, roughly 27% of the proliferative activity was still evident at 48 hours, compared with that of the control cultures. This is in contrast to the U373MG cell line in Fig. 2, B, in which even 0.1 n*M* kills more than 95% (<5% proliferative activity compared with control cultures was evident) in 72 hours. In comparison, Fig. 2, A, shows that at 72 hours, 10 n*M* DTAT inhibited statistically significantly (P = .005) more (94%) of the U118MG cells than did 10 n*M* DT-IL-13. These findings indicated that *in vitro,* DTAT inhibited the U118MG glioblastoma better than did DT-IL-13. These differences were obvious even at 48 hours (P<.001).

DTAT Selectivity *In Vitro*

To study the selectivity of DTAT, it was first tested against various cell lines such as the B cell line Daudi and breast cancer line SKBR3 that do not express uPAR. Fig. 3, A, shows that growth of U87MG was inhibited by DTAT (IC_{50}<1 n*M*). In contrast, the growth of Daudi and SKBR3 cells was not inhibited. The growth of the murine neuroblastoma cell line Neuro-2a was slightly inhibited at the highest concentration (10 n*M*). To further study selectivity in a different experiment, U118MG cells were treated with an irrelevent FP control (Fig. 3, B). DT-m-IL-4 was used as a negative control, because mouse IL-4 is species-restricted and does not bind to human cells. Whereas DTAT inhibited U118MG cells (Fig. 2, A), DT-m-IL-4 had no effect. Fig. 3, A and B, indicates that DTAT is highly selective in its ability to kill uPAR-expressing cells.

To determine whether exposure of DTAT to its target for longer periods would heighten its ability to kill, DTAT was incubated with its U118MG target for periods of 24, 48, and 72 hours. Dose–response studies revealed that the maximum level of cytotoxicity was obtained after 48 hours (data not shown). A longer exposure of 72 hours did not enhance the cytotoxicity.

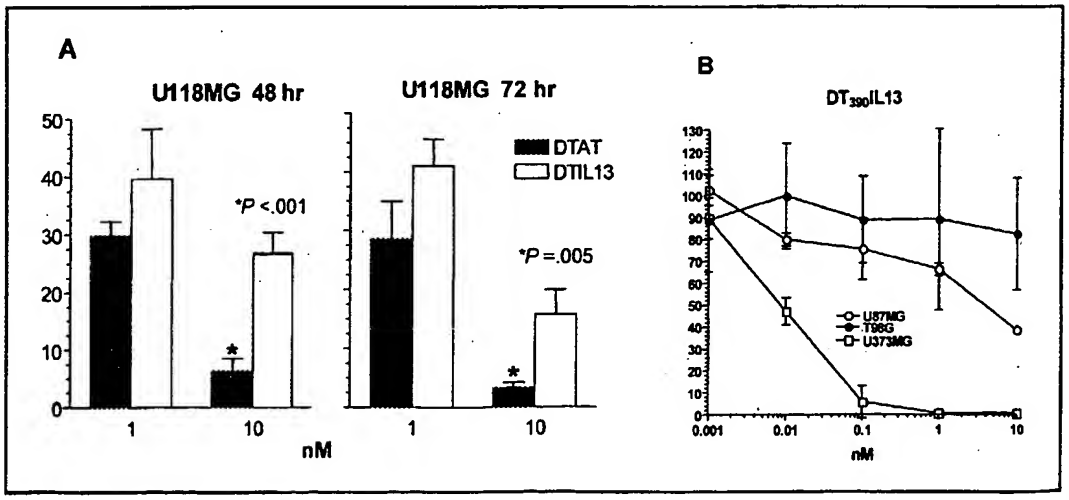

Fig. 2. A) Activity of DTAT (diphtheria toxin [DT]-amino terminal [AT] fragment of urokinase-type plasminogen activator [uPA]) and $DT_{390}IL13$ (DTIL13) against the U118MG human glioblastoma cell line. Cells were cultured with the fusion protein (FP) for 48 hours (**left panel**) or 72 hours (**right panel**), and incorporation of tritiated thymidine was then assayed. Data are expressed as percent control response versus concentration in nanomoles (n*M*). Control counts per minute (cpm) values (mean + 1 standard deviation [SD]) for U118MG (without treatment) were 9893 ± 343 after 48 hours and 13 151 ± 859 cpm after 72 hours. Each **bar** on the graph represents the mean of triplicate samples, and the **error bars** represent the upper limit of the 95% confidence intervals (CIs). The proliferation of cells treated with 10 m*M* DTAT was statistically significantly inhibited in comparison with that in cells given 10 m*M* DTIL13 at 48 hours (P<.001) and at 72 hours (P = .005). B) Activity of DT_{390} amino acid segment fused with interleukin 13 ($DT_{390}IL13$) against the human glioblastoma cell lines U87MG, T98G, and U373MG. Glioblastoma cells were cultured with FP and after 72 hours, tritiated thymidine incorporation was assayed as a measure of tumor cell proliferation. Data are expressed as a percentage of control response, where the controls are not treated with FP. Control (cpm + 1 SD) values for U87MG, T98G, and U373MG were 40 481 ± 2792, 90 384 ± 1771, and 37 834 ± 2005, respectively. Data are expressed as a percentage of average control response versus concentration of FPs in nanomoles (n*M*). Each point on the graph represents the mean of triplicate readings ± 95% CI.

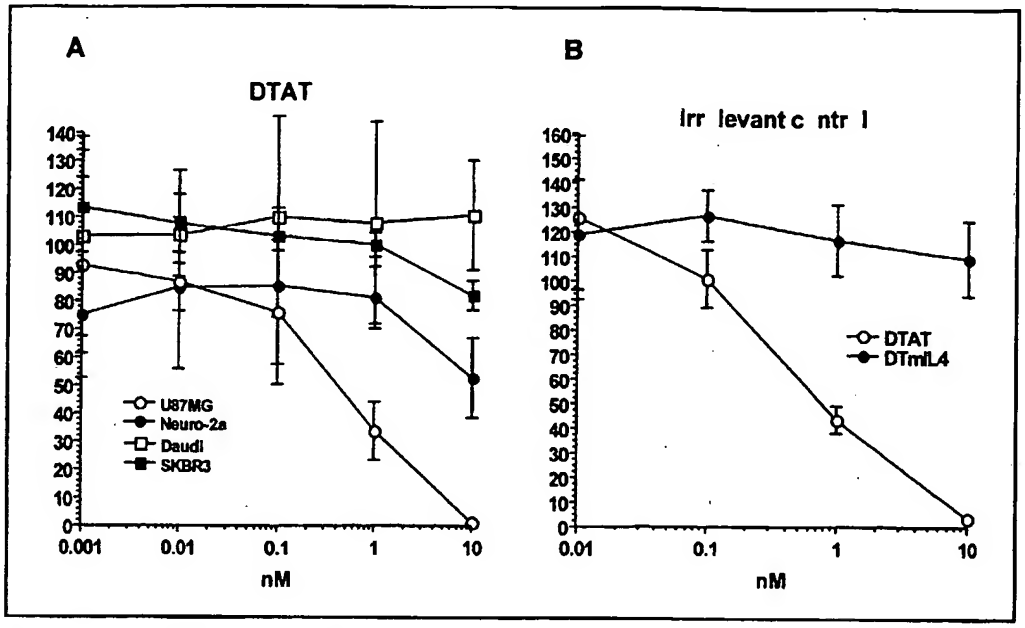

Fig. 3. A) Activity of DTAT (diphtheria toxin [DT]-amino terminal [AT] fragment of urokinase-type plasminogen activator [uPA]) against various cell lines: U87MG (human glioblastoma line), Neuro-2a (murine neuroblastoma line), Daudi (derived from human Burkitt's lymphoma), and SKBR3 (human mammary gland adenocarcinoma line). Cells were cultured with fusion protein (FP) and, after 72 hours, incorporation of tritiated thymidine was assayed. Data are expressed as a percentage of control response. Control counts per minute (cpm) values (± 1 standard deviation [SD]) for U87MG, Neuro-2a, Daudi, and SKBR3 cells were 35 162 ± 1104, 16 445 ± 1116, 15 529 ± 2691, and 99 320 ± 6452, respectively. Each point on the graph represents the mean of triplicate readings ± 95% confidence intervals. B) Activity of irrelevant control FP DTmIL4 (DT-mouse interleukin 4) against U118MG cells. Cells were cultured with FP and tritiated thymidine incorporation was assayed after 72 hours. Control value (cpm ± 1 SD) for U118MG was 9928 ± 553.

We concluded that a near maximum level of killing was reached after 48 hours of exposure to DTAT (data not shown).

Ability of DTAT to Kill Endothelial Cells

As mentioned earlier, the ability of DTAT to bind to the tumor and its microvasculature could provide a therapeutic advantage because tumor growth is dependent on a thriving vasculature. To measure the binding ability of DTAT, DTAT was incubated with human HUVEC *in vitro*. Fig. 4, A, shows that DTAT was able to inhibit the proliferation of HUVEC in a dose-dependent manner with an IC_{50} of approximately 2 n*M*. In contrast, a number of control FPs (binding those receptors not found on HUVECs, including mouse IL-4 receptor [m-IL-4R], human IL-2 receptor [h-IL-2R], human IL-13 receptor [h-IL-13R], or mouse IL-3 receptor [m-IL-3R]) were not inhibitory.

To determine whether the activity of DTAT on HUVECs was mediated by the ATF, polyclonal anti-urokinase antibody was pre-incubated with DTAT before adding to cultured HUVECs (Fig. 4, B). Anti-urokinase blocked the inhibition by 1 and 10

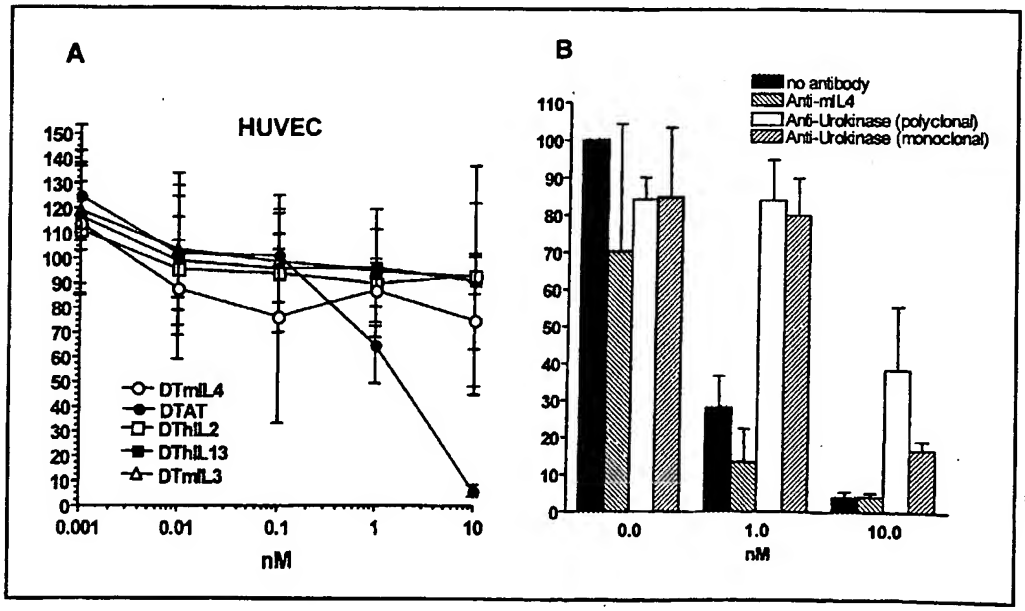

Fig. 4. A) Activity of DTAT (diphtheria toxin [DT]-amino terminal [AT] fragment of urokinase-type plasminogen activator [uPA]) and various control fusion proteins (FPs) against human umbilical vein endothelial cells (HUVEC). HUVEC were cultured with FP and, after 72 hours, incorporation of tritiated thymidine was assayed as a measure of tumor cell proliferation. Data are expressed as percent control response, where the controls are not treated with FP. Control count per minute (cpm) ± 1 standard deviation (SD) for HUVEC was 87 969 ± 7798. B) Ability of anti-urokinase antibody to block the binding of DTAT to HUVEC. Cells were cultured with DTAT for 72 hours in the presence or absence of monoclonal or polyclonal anti-urokinase antibody or control anti-mouse interleukin 4 (mIL4) antibody. Then, tritiated thymidine incorporation was assayed. Control cpm ± 1 SD value for HUVEC was 47 620 ± 907.

n*M* DTAT. A control anti-mouse IL-4 antibody (11B11) had no effect on DTAT activity.

Together, these data show that DTAT selectively kills endothelial cells *in vitro* through its ability to bind uPAR.

In Vivo Anti-Tumor Activity of DTAT Measured Against U118MG Cell-Induced Tumors in Nude Mice

To determine the effectiveness of DTAT, we established a nude mouse model of human glioblastoma. U118MG tumor cells were inoculated into nude mice. When the tumor had established on day 30, mice were given a course of DTAT. Fig. 5, A, shows that in a group of five mice, tumors that had reached the size of 0.2 cm^3 all regressed following a 5-dose course of DTAT with 20 mg/day given every other day. In contrast, tumors in groups of mice treated with control DT-h-IL-2 and PBS did not regress over the study period. Tumors in these groups continued to increase in size. There was a statistically significant difference (P = .05) in tumor growth curves on day 48 when the DTAT group was compared with the control groups. Also, DT-IL-13 did not inhibit the growth of U118MG-induced tumors *in vivo*. Thus, *in vitro* findings were similar to the *in vivo* results. These results also supports the idea that DTAT might be a useful treatment of tumors that are not effectively treated with DT-IL-13.

Fig. 5, B, shows the tumor growth curves of each of the individual mice in the DTAT group. The tumors steadily diminish in size following DTAT treatment. Only one of the tumors did not entirely regress by day 65. These *in vivo* findings were reproduced in another experiment, again with five mice in each experimental group.

Effects of DTAT on Body Organs

To study the toxic effects of the tumor treatment dose of DTAT, C57BL/6 mice without tumors were given the same 5-dose course of DTAT with 20 mg/day given every other day by subcutaneous treatment as the tumor-bearing nude mice and then studied the day following the last injection. Frozen tissue sections were stained with H & E and examined microscopically. Functional assays were performed on serum. Kidney tissue appeared unaffected by DTAT treatment with the exception of some minor neutrophil infiltration. Fig. 6, A, shows that glomeruli appeared healthy. These studies were particularly important because similar doses of FP in other studies *(28)* have induced glomerular destruction, rupture of renal tubules, and proximal tubular vacuolization. Liver effects were studied in the same group of mice (Fig. 6, B). Examination of tissues revealed a few areas of infiltration of peripheral mononuclear cells and neutrophils. Otherwise, tissues appeared relatively unaffected by treatment. Fig. 6, B (inset), shows that DTAT had no effect on heart; it also had no effect on the spleen (not shown). Minor neutrophil infiltration was observed in the lung compared with normal controls (not shown).

Functional analysis of tissue revealed a statistically significant (P = .046), albeit non-life-threatening, elevation in ALT level in serum (Fig. 7, A). Although these elevations were not indicative of liver failure and histology indicated that the liver was intact, it appeared that DTAT did affect the liver at this dose. To further study the effects of DTAT on kidney function, serum was analyzed from these same mice. Fig. 7, B, shows no significant fluctuations in BUN levels following DTAT treatment, indicating that treatment did not interfere with renal activity. As a positive control, a group of mice were treated with DTanti-CD3sFv, a fusion toxin that has been shown to mediate renal damage in past studies *(28)*. This positive control caused a statistically significant (P = .033) elevation in BUN activity. Histology studies confirmed glomerular damage and tubular rhexis induced by DTanti-CD3sFv.

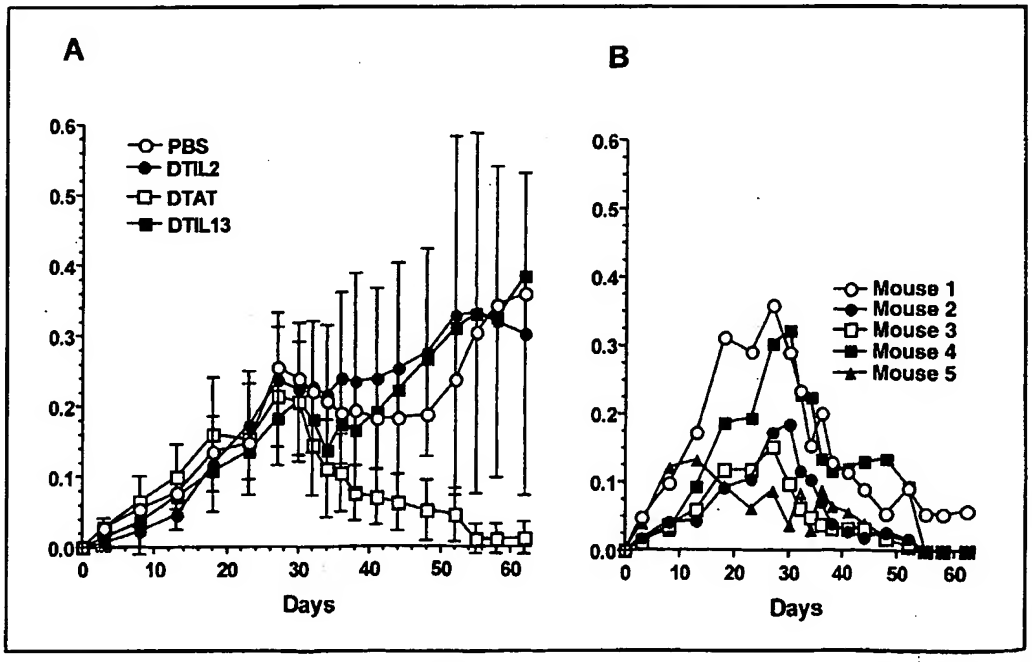

Fig. 5. DTAT (diphtheria toxin [DT]-amino terminal [AT] fragment of urokinase-type plasminogen activator [uPA]) administered intratumorally inhibits the growth of established human U118MG tumors in nude mice. U118MG tumor cells were administered subcutaneously to nude mice (n = 5 per group). Five injections of 20 μg/day DTAT in 50 μL of phosphate-buffered saline (PBS) beginning on day 28 were given every other day over a period of 9 days. DTAT was injected from three different directions by using a 0.5-cc insulin syringe with a 28-gauge needle over a period of approximately 1 minute. A) Tumor size was monitored approximately three times per week, and tumor volume (cm^3) was plotted versus time. Mice were treated with DTAT, DT-interleukin 13 (DTIL13), DT-interleukin 2 (DTIL2), or PBS. For DTIL2 and DTIL13, five injections of 10 μg/dose were given. Tumor growth was statistically significantly (P = .05) decreased in DTAT-treated mice as compared with PBS-treated control mice by Student's t test. Error bars represent ± the 95% confidence interval. B) Tumor growth curves for the five individual mice given DTAT (presented as a group average in Fig. 5, A).

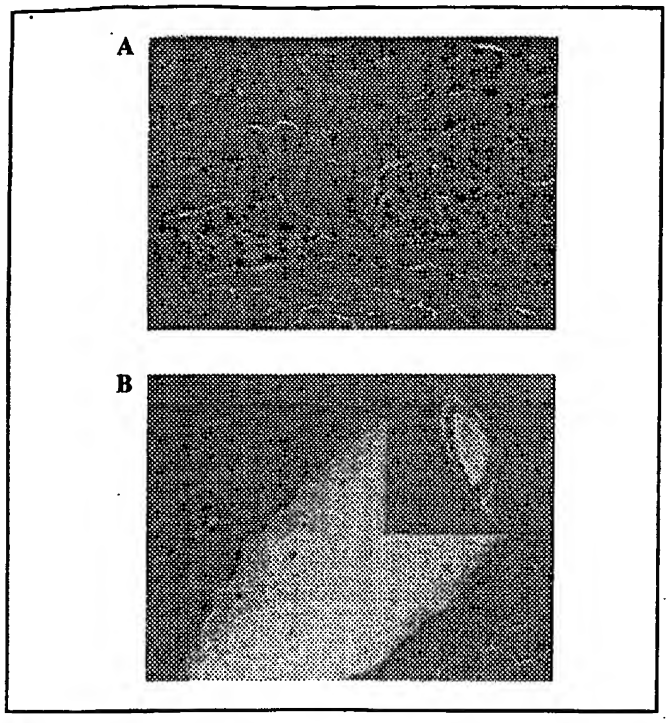

Fig. 6. Histologic study of kidney (**A**), liver (**B**), and heart (**B, inset**) from DTAT (diphtheria toxin [DT]-amino terminal [AT] fragment of urokinase-type plasminogen activator [uPA])-treated mice. A group of normal C57BL/6 mice without tumor were treated in the same manner as the mice described in Fig. 5. Mice were given five 20-μg/dose injections of DTAT subcutaneously. Organs were removed, sectioned, and stained with hematoxylin and eosin to visualize organ damage. Three animals per group were examined with identical results.

Discussion

An original contribution of these studies is the description of DTAT, a diphtheria-toxin-based FP directed against the uPAR overexpressed on glioblastoma cells. Previous studies by other investigators *(11,31)* have encouraged the development of new drugs targeting the uPAR, but we provide evidence for the first time that uPAR may serve as a valid target for FP-directed therapy against chemotherapy and radiation refractory glioblastoma. An important element of these studies was the use of the nude mouse model to assess the effect of DTAT on glioblastoma independently of effects on the tumor neovasculature. DTAT, which is not species cross-reactive *(32)*, inhibited the human glioblastoma U118MG but could not be used to assess the anti-endothelial cell effects because tumor vasculature was derived from the mouse, not from humans. Still, it appears that DTAT, even without the help of an antivascular effect, is delivering potent therapy and killing glioblastoma tumors in this model. This is also the first time that attachment of the catalytic domain of DT and its translocation-enhancing region has been used to enhance the ability of ATF to internalize and serve as a potent and selective IT. Clearly, these findings raise some important questions regarding the deployment of this agent for clinical use.

Interestingly, intratumoral administration of DTAT caused the regression of tumors, even after dosing was discontinued. Ramakrishnan et al. *(18)* reported that an immunotoxin made by chemically conjugating vascular endothelial growth factor (VEGF) to truncated diphtheria toxin successfully inhibited the growth of subcutaneous tumors in nude mice. In these studies, tumor growth in the treated group was delayed as a consequence of the VEGF FP treatment. When treatment was stopped, the tumor immediately began to grow. This difference may be due to the intratumoral route of administration of DTAT as compared with systemic delivery in their study. Less than 0.001% of a systemically administered biologic agent (such as an FP) distributes to the tumor *(5)*. Thus, direct intratumoral therapy of glioblastoma, with an agent that binds both tumor and vasculature, may be an advantage in treating glioblastoma that seldom leaves the cranium. However, there could be other explanations for the differences, including the fact that two different model systems were used or that the two different FPs may be internalized differently.

Enzyme studies revealed that the dose of DTAT (5 doses × 20 μg/dose) that caused tumor regression had some effect on the liver but not on other organs of normal mice. It is possible that further elevations in dosage might be prohibitive. However, our

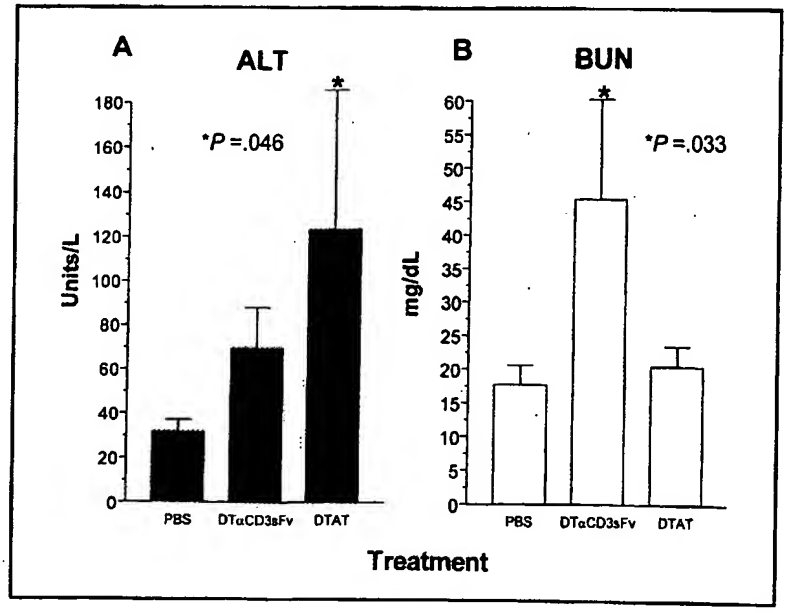

Fig. 7. C57BL/6 mice were randomly grouped (n = 5) and injected with DTAT (diphtheria toxin [DT]-amino terminal [AT] fragment of urokinase-type plasminogen activator [uPA]), phosphate-buffered saline (PBS), or a control DTanti-CD3sFv fusion toxin. DTanti-CD3sFv has been previously shown to cause organ toxicity. On the day immediately following the last injection, mice were bled, and individual serum samples were studied for alanine aminotransferase (ALT) (**panel A**) and blood urea nitrogen (BUN) (**panel B**). Data were averaged. Groupwise comparisons were performed with Student's *t* test. The elevation in ALT in the DTAT-treated group in **panel A** (P = .046) and the elevation in BUN in the DTanti-CD3sFv-treated group in **panel B** (P = .033) were statistically significant as compared with PBS controls. U/L = units/liter; mg/dL = milligram/deciliter.

toxicity studies were performed in a different model by giving normal mice without tumors subcutaneous injections. Thus, higher doses of DTAT may be tolerated in tumor-bearing mice because the tumor combined with its extensive vascular network could provide an expansive "antigenic sink" that absorbs injected DTAT and limits the amount that would leave the tumor and traffic to nontarget sites such as liver and kidney. In fact, we have recently found (Vallera DA, Buchsbaum DJ: unpublished data) that radiolabeled FP was remarkably localized in the tumor over time when FP was administered intratumorally. Twenty-four hours after administration, 12% of the injected dose/gram (ID/g) was found in glioblastoma, whereas less than 1% was found in kidney and none was found in the blood. In mice without tumor, the absence of tumor and this antigenic sink may put more toxic stress on the liver, which would explain our ALT elevations. Interestingly, we already have preliminary evidence that this may be the case, because mice with large (>6 cm^3) subcutaneous tumors tolerated a 100 μg/dose course of DTAT, and the tumor underwent a 50% reduction in size. All together, this also indicates a reasonable therapeutic window that makes DTAT attractive for therapeutic use.

The difference in renal toxicity and liver toxicity of DTanti-CD3sFv and DTAT is difficult to explain. We do know that dimerizing DTanti-CD3sFv prevents the renal toxicity by preventing its filtration into the kidney *(27)*. Size cannot be the only issue because DTAT is smaller than monomeric DTanti-CD3sFv but is not as toxic to the kidney. Since DTAT is not reactive with murine uPAR, DTAT toxicity is not likely to be attributed to specific activity. Since DTanti-CD3sFv is reactive with murine T cells, it is possible that when it is filtered into the kidney, DTanti-CD3sFv targets itinerant T cells. Perhaps, their destruction in the kidney promotes damage. It is also possible that because the DT moiety is identical in the two agents, toxicity differences are somehow attributable to physical differences in the ligand moiety of the molecules. Other DT-containing FPs directed to different cell surface determinants, such as CD25 and the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor, have exhibited renal and liver toxicity in rodents and in humans *(33,34)*.

Future studies will emphasize toxicity in normal brain. Because DTAT cross-reacts with certain primate species *(35)*, it could be tested in normal monkey brain. However, tumor cannot be grown in monkeys, and the presence of tumor may affect the outcome of DTAT administration. A better model might be a syngeneic mouse model in which we could intracranially implant mouse glioblastoma. Because mouse and human DTAT are not cross-reactive, we would assemble and purify murine DTAT. Unlike our current nude mouse model, murine DTAT could be injected into the brain of mice with syngeneic tumor and would bind to both tumor and its neovasculature.

Why would we expect that DTAT would kill endothelial cells in tumor vasculature and spare normal endothelial cells? First, DTAT may be selective for the rapidly expanding tumor endothelium as compared with normal endothelium. Thus, uPAR expression may be differentially high in the proliferating endothelium of the tumor neovasculature, although uPAR has not been convincingly demonstrated to be a proliferation-associated marker. Second, alteration in the endocytic/processing pathway of internalized cytotoxic conjugate has been observed and suggested as the mechanism to account for the differential sensitivity of endothelial cells to VEGF–toxin conjugates *(36)*. The same could be true for the intracellular processing of DTAT, which has not yet been studied. Third, immunohistochemical studies revealed that uPAR is mostly expressed in tissues undergoing extensive remodeling, e.g., in trophoblast cells in the placenta, in keratinocytes at the edge of incisional wounds, and in cells of primary tumor and metastasis *(37)*. Perhaps the more quiescent normal endothelium is not as readily affected by treatment.

The literature supports IL-13R and IL-4R, which share receptor components *(8)*, as useful targets for immunotoxin therapy in brain tumors *(9,30,38,39)*. However, DTAT was developed as an alternative for tumors that fail to respond to DT-IL-13 treatment. On the basis of the literature *(30)*, IL-13R is also highly expressed on some brain tumors but minimally expressed on others; thus, IL-13-FP may show promise in certain instances. However, GBM is heterogeneous and some glioblastomas may not respond to DT-IL-13 either because they don't express IL-13R or because it is not expressed in high enough density. DTAT represents a useful alternative therapy for these tumors. With two different FPs, it may also be possible to use combination therapy because some tumors may simultaneously express both receptors. In this instance, combinations of FPs may bind higher numbers of receptors on the cell surface. Presumably more toxin will be internalized. We have previously reported that kinetics of cell kill was far greater with FP combinations than kinetics observed with individual FP *(40)*. More recently, the Vitetta group *(41)* observed that anti-CD19 and anti-CD22 IT are more effective against patient-derived B-acute lymphoblastic leukemia cells and found the combination to be more effective than the individual FP. Combination studies are under way.

Why did we target uPAR? Several tumor cell lines overexpress uPAR, including breast cancer *(12,13)*, melanoma *(14)*, colon cancer *(15)*, and prostate carcinoma cells *(16)*, and the literature describing its expression on glioblastoma is extensive *(11)*. Others have targeted uPAR with FP *(20,42)*. To simplify our approach, we cloned the ATF of uPA. This molecule binds to uPAR-expressing cells *(19)*. However, the ATF domain completely lacks the catalytic domain of uPA but possesses an EGF-like domain that comprises the receptor binding sequence of human uPA. Therefore, ATF can target cell surface molecules of uPAR.

Glioblastoma therapy affords the advantage of local delivery, and clinical responses for FP therapy for this disease have exceeded responses for FP in any other disease. Response rates in excess of 50% have been reported *(7)*. In the first GBM FP phase I clinical study, Youle's group at the National Institutes of Health (NIH) *(43)* evaluated the toxicity of an FP consisting of transferrin as a ligand linked to a mutated form of diphtheria toxin called CRM107. A trial was conducted with regional therapy of Tf-CRM107 in 18 patients. At least a 50% decrease in tumor volume occurred in nine of 15 assessable patients. Reduction occurred no earlier than a month after completion of the first Tf-CRM107 infusion, and the response did not peak in four patients until 6–14 months after the first treatment. The median survival after treatment in the group of nine responders who had malignant glioblastomas was 74 weeks (three were still alive at 102–142 weeks after the first treatment compared with 36 weeks for the nonresponders). These findings are impressive and dramatic for a disease that is extremely difficult to treat. Similar results have been reported for leptomeningeal carcino-

matosis *(44)*. Rand et al. *(38)* have investigated the safety and activity of directly infusing an IL-4-FP made by linking circularly permuted IL-4 and truncated *Pseudomonas* toxin, also with promising results.

In summary, DTAT is highly selective and causes the regression of human glioblastoma in a mouse model without undue toxicity. Because it is different from other brain tumor targeting agents in its ability to directly target glioblastoma while simultaneously targeting the vasculature, it should be pursued as a useful alternative for treatment of chemotherapy-resistant and radiation-resistant glioblastomas. Studies in the nude mouse have been informative but are somewhat limited, because human DTAT is not species cross-reactive. Thus, the effect of DTAT on tumor can be assessed but not its reactivity on tumor microvasculature derived from the mouse. The construction of a DTAT homologue that is reactive with mouse endothelial cells is currently under way.

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NOTES

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Endogenous Sex Hormones and Breast Cancer in Postmenopausal Women: Reanalysis of Nine Prospective Studies

The Endogenous Hormones and Breast Cancer Collaborative Group

Background: **Reproductive and hormonal factors are involved in the etiology of breast cancer, but there are only a few prospective studies on endogenous sex hormone levels and breast cancer risk. We reanalyzed the worldwide data from prospective studies to examine the relationship between the levels of endogenous sex hormones and breast cancer risk in postmenopausal women.** ***Methods:*** **We analyzed the individual data from nine prospective studies on 663 women who developed breast cancer and 1765 women who did not. None of the women was taking exogenous sex hormones when their blood was collected to determine hor m ne levels. The relative risks (RRs) for breast cancer associated with increasing hormone concentrations were estimated by conditional logistic regression on case–control sets matched within each study. Linear trends and heterogeneity f RRs were assessed by two-sided tests or chi-square tests, as appropriate.** ***Results:*** **The risk for breast cancer increased statistically significantly with increasing concentrations of all sex hormones examined: total estradiol, free estradiol, n n-sex hormone-binding globulin (SHBG)-bound estradiol (which comprises free and albumin-bound estradiol), estrone, estr ne sulfate, androstenedi ne, dehydroepiandroster ne, dehydroepiandroster ne sulfate, and testosterone. The RRs f r women with increasing quintiles f estradiol concentrati ns, relative t the l west quintile, were 1.42 (95% confidence interval [CI] = 1.04 to 1.95), 1.21 (95% CI = 0.89 to 1.66), 1.80 (95% CI = 1.33 to 2.43), and 2.00 (95% CI = 1.47 to 2.71; P_{trend}<.001); the RRs for women with increasing quintiles of free estradiol were 1.38 (95% CI = 0.94 to 2.03), 1.84 (95% CI = 1.24 to 2.74), 2.24 (95% CI = 1.53 to 3.27), and 2.58 (95% CI = 1.76 to 3.78; P_{trend}<.001). The magnitudes of risk associated with the other estrogens and with the androgens were similar. SHBG was associated with a decrease in breast cancer risk (P_{trend} = .041). The increases in risk associated with increased levels of all sex hormones remained after subjects who were diagnosed with breast cancer within 2 years of blood collection were excluded from the analysis.** ***Conclusion:*** **Levels of endogenous sex hormones are strongly associated with breast cancer risk in postmenopausal women. [J Natl Cancer Inst 2002;94:606–16]**

Breast cancer risk is partially determined by several hormone-related factors, such as age at menarche, parity, and age at menopause, and it has long been hypothesized that high levels of

Correspondence to: Timothy J. Key, D.Phil., Endogenous Hormones and Breast Cancer Collaborative Group, Cancer Research U.K. Epidemiology Unit, University of Oxford, Gibson Bldg., Radcliffe Infirmary, Oxford OX2 6HE, U.K. (e-mail: Tim.Key@cancer.org.uk).

See "Appendix" for affiliations of the Endogenous Hormones and Breast Cancer Collaborative Group.

See "Notes" following "References."

STIC-ILL

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From: Canella, Karen
Sent: Thursday, February 13, 2003 6:15 PM
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Telephone Number 308-8362

Application Number 10/033,577

1. Research in Microbiology, 1996 Nov-Dec, 147(9):697-705

Res. Microbiol.
1996, 147, 697-705

Contribution of plasminogen activator urokinase to *in vitro* cytotoxicity of diphtheria toxin

C. Guidi-Rontani (*)

Unité des Toxines microbiennes, CNRS URA 18580, Institut Pasteur, 75724 Paris

SUMMARY

Nicking of diphtheria toxin (DT), i.e. proteolytic cleavage at an arginine-rich region within the first disulphide loop, is a prerequisite to the intoxication process. We show that protease(s) required in this process was synthesized and secreted by the sensitive cells and that antibodies against plasminogen activator urokinase (uPA) decreased the *in vitro* cytotoxicity of DT on Vero cells. Our results demonstrate that uPA secreted by Vero cells cultured *in vitro* is one of the cellular proteases involved in the cleavage and activation of diphtheria toxin.

Key-words: Toxin, *Corynebacterium diphtheriae,* Urokinase; Plasminogen activator, Cytotoxicity.

INTRODUCTION

Diphtheria toxin (DT) is a 58.342-kDa protein synthesized as a single polypeptide and secreted extracellularly as a 535-amino-acid-residue polypeptide, a proenzyme, by *Corynebacterium diphtheriae* (Collier and Kandel, 1971; Drazin *et al.*, 1971; Gill and Dinius, 1971; Greenfield *et al.*, 1983) lysogenic for bacteriophage ß tox$^+$ (Freeman, 1951). The crystal structure at 2.5 Å resolution revealed a three-structural domain molecule (Choe *et al.*, 1992). The protein is toxic to most eucaryotic cells and species (rats and mice are insensitive) and the effect of the toxin on cells is to inhibit protein synthesis (Brown and Bradley, 1979). The intoxication process consists of the following (Collier, 1975; Pappenheimer, 1977): (i) binding to a cell surface membrane protein representing the precursor of a heparin-binding epidermal growth factor(EGF)-like growth factor (Naglich *et al.*, 1992); (ii) receptor-mediated endocytosis and intracellular transport; (iii) translocation of the enzymatically active components (A fragment) of the toxin into the cytosol; and (iv), once in the cytoplasm, enzymatic catalysis by the A fragment of the transfer of the ADP-ribosyl moiety of NAD^+ to a unique posttranslationally modified histidine residue, diphthamide (2-(3-carboxyamido-3-(trimethylammonio)propyl)histidine) (VanNess *et al.*, 1980), within elongation factor 2 (Collier, 1967), inactivating the factor, thereby arresting protein synthesis and consequently causing cell death.

It has been shown that diphtheria toxin, as a single polypeptide chain, is fully cytotoxic but lacks ADP-ribosyltransferase activity *in vitro*. For

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(*) Present address: Laboratoire de Génétique moléculaire des Toxines, CNRS URA 18580, Institut Pasteur, 75724 Paris, France.

in vitro enzymic activity, intact DT must undergo two covalent alterations in structure: mild treatment of the toxin with proteolytic enzymes such as trypsin (this form of toxin is termed "nicked" toxin (Drazin *et al.*, 1971)), followed by reduction with thiols. Nicked toxin is split into two large peptides linked by a disulphide bond (Collier and Kandel, 1971); these have been designated as A fragment (Mr 21,167) and B fragment (Mr 37,199). This proteolytic action occurs within an arginine-rich sequence (190-194) that is subtended by a disulphide bond involving cysteine residues at position 186 and 201 about one third of the length of the molecule from the amino terminus. This A fragment, corresponding to the N-terminal position of the toxin (Uchida *et al.*, 1973), catalyses the ADP ribosylation of mammalian elongation factor 2 (Drazin *et al.*, 1971; Gill and Pappenheimer, 1971), whereas B fragment is involved in receptor binding to the precursor of a heparin-binding EGF-like growth factor.

From the study of DT mutants altered in the interfragment region by site-directed mutagenesis (Ariansen *et al.*, 1993), the study of DT/IL2 fusions (Williams *et al.*, 1990) and the study of ricin A/protein A fusions carrying the DT loop (O'Hare *et al.*, 1990), it is clear that cleavage of intact DT in the 14-amino acid loop is important for cytotoxicity (Gordon and Leppla, 1994). The protease-sensitive region contains the amino acid sequence Arg-Val-Arg-Arg at residues 190-193. Thus, *in vivo* limited proteolysis of the intact DT molecule is an essential step in intoxication, and proteolysis would occur *in vivo* during the process of internalization of the toxin by cells. A major point of interest in the lethal action of toxins on cells is the process by which the enzymatically active protein undergoes transfer across membranes and is liberated into the cytosol. While studies demonstrated that DT underwent receptor-mediated endocytosis (Keen *et al.*, 1982) and required correct intracellular routing, the exact process of activation of DT and the precise mechanism of the translocation step itself are still currently poorly understood. This process involved exposure to low pH (pH 5.3) (Moskaug *et al.*, 1988), protease processing and disulphide bond activation (Draper and Simon, 1980; Sandvig and Olsnes, 1980). Acidic pH within early acidic endosomes of sensitive cells triggers a conformational change in DT that is believed to cause the B fragment to be inserted into the vesicular membrane and the enzymic A fragment of cleaved DT to be released into the cytosol. In the present work, we have addressed the question of the identity of protease(s) required for *in vivo* activation of diphtheria toxin.

MATERIALS AND METHODS

SDS-PAGE

Proteins were analysed by sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970). Gels were stained with Coomassie brilliant blue R-250 (Fairbanks *et al.*, 1971). The molecular weight was estimated with standard protein markers as references.

Zymographic analysis of proteinases

This procedure was used to identify proteinase activity according to Heussen and Dowdle (1980). Proteases were analysed by 11% polyacrylamide gels containing 0.1% (w/v) SDS cast with 0.1% (w/v) copolymerized gelatin and in the presence or absence of copolymerized plasminogen (15 μg ml) as sequential substrates. The samples were loaded onto the gel without boiling or treatment with ß-mercaptoethanol. After electrophoresis at 4°C at a constant current of 8 mA, the gel was soaked in 2.5% (v/v) Triton-X100 in water at room temperature for 1 h to remove SDS and to restore enzymic activity. Then the gel was transferred to a bath containing 0.1 M glycine-NaOH pH 8.3 and incubated at 37°C for 3 h. Finally, the gels

DMEM = Dulbecco's minimal essential medium.
DT = diphtheria toxin.
EGF = epidermal calf growth factor.
FCS = foetal calf serum.
IL = interleukin.
MW = molecular weight.
PA = plasminogen activator.
PBS = phosphate-buffered saline.
TCA = trichloroacetic acid.
uPA = PA urokinase.
uPAR = uPA receptor.

were fixed and stained in 0.1% (w/v) of amido black in methanol/acetic acid/water (30:10:60).

Cells and cell cultures

A Vero cell line (African green monkey kidney cell line) was maintained as monolayers and cultured in DMEM supplemented with 50 units penicillin/ml, 50 µg streptomycin ml and 1 mM L-glutamine with or without 2% (v/v) foetal calf serum (FCS) in an atmosphere containing 5% CO_2. B4, a human melanoma cell line, was maintained indefinitely under serum-free conditions (gift from Dr. E.L. Wilson, Dept. of Clinical Science and Immunology, University of Cape Town, Cape Town, South Africa) The day before use, the cells were seeded into 24-well disposable trays in the same medium at a density of approximately 50,000 cells/well. The doubling time of the cells was 36 h.

Cytotoxicity assay

Toxicity assays were performed with Vero or B4 in multi-well tissue culture plates. Medium was removed from cells growing in 24-well disposable trays, and 0.5 ml leucine-free medium, to which had been added 0.5 µCi ^{3}H-leucine/ml or 1 µCi ^{3}H-thymidine/ml, was added to each well. After 1 h at 37°C, the medium was removed and the cells were treated twice with cold 10% (w/v) trichloroacetic acid (+ 1 mg unlabelled leucine/ml), rinsed once with PBS and dissolved in 0.4 ml of 0.1N NaOH at room temperature for 5 min. Incorporation of leucine or thymidine into TCA-precipitable material was determined by liquid scintillation.

RESULTS AND DISCUSSION

Highly purified unnicked DT toxin was essential for the realization of this study. In purified toxin preparations, the "nicked" toxin is found in variable proportions. Therefore, in order to obtain "totally" unnicked toxin, we carried out purification steps of DT preparation according to the method of Middlebrook and Leatherman (1982). The purified preparation of DT, composed of 99% pure but 75% unnicked protein, was treated with 0.5 M guanidine hydrochloride and 0.1 M dithiothreitol for 30 min at 25°C. The material was dialysed against Hanks' balanced salt solution. Under these conditions, the A and B fragments of the nicked toxin did not reassociate. The material obtained was gel-filtered by fast protein liquid chromatography on a "Superose-12" column (type HR 10/30, Pharmacia, Uppsala, Sweden). DT molecules were uncleaved, corresponding to the intact, native toxin. This method gave an extent of nicking estimated to be less than 5%.

To assess an eventual contribution of exogenous protease(s) in the activation of DT, we first chose to test the cytotoxicity of intact DT on the most sensitive cell line, Vero, an African green monkey kidney cell line, in the presence or absence of serum. We tested the eventual contribution of FCS in the cytotoxicity of unnicked DT by measuring the rate of protein synthesis in cultured cells. At a density of approximately 150,000 cells/well, this cell line was washed with media to remove unattached cells and 0.5 ml/well fresh medium was added (DMEM supplemented with 50 units penicillin/ml, 50 µg streptomycin/ml, 1 mM L-glutamine with or without 2% (v/v) FCS serum) with unnicked DT (10^{-13} to 10^{-8} M). After 20 h, the medium was removed and protein synthesis was assessed with a 1-h pulse of 1 µCi of ^{3}H-leucine/ml at 37°C in 0.5 ml of medium containing low level leucine (1/20, the concentration present in the original medium). All experiments described in this report were done in duplicate cells seeded in 24-well plates. Controls included the observation of the cells under phase-contrast light microscope. As can be seen in figure 1, cytotoxic effects of unnicked DT with or without FCS were strikingly similar on Vero cells. ID_{50} occurred at a concentration of 5×10^{-12} M unnicked DT toxin in both cases (ID_{50}: the concentration of toxin that inhibited protein synthesis by 50% relative to untreated control). *In vitro,* unnicked DT had a similar potency both in the absence and in the presence of FCS. On the other hand, a similar result was observed with B4, a human melanoma cell line, which can be maintained indefinitely under serum-free conditions (data not shown). No detectable contribution of the serum could be observed in these experiments, allowing one to exclude a mandatory contribution of exogenous protease present in serum in the *in vivo* activation process of DT. This result provided a clear indication that host protease(s) required in the activation of DT must be synthesized by sensitive cells.

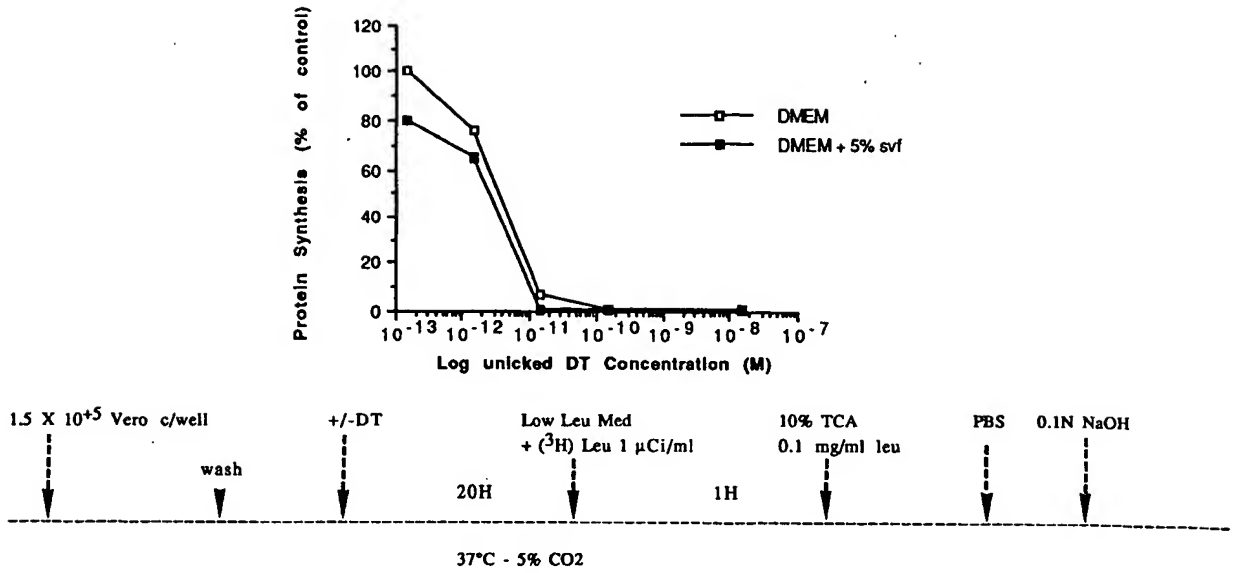

Fig. 1. Cytotoxicity of unnicked DT in the absence or presence of FCS.

Increasing amounts of the unnicked DT were added to cultures of Vero cells in the absence or presence of 5% FCS. The results were expressed as percent of the control values. The ^{3}H-leucine incorporation of non-toxin-treated cells ranged from 10,000 to 19,000 cpm.

Having determined that the protease(s) had to be produced by the sensitive cells, we investigated the nature of the protease that were involved in DT activation. To this end, well-characterized proteinase inhibitors such as antipain, leupeptin, chymostatin, pepstatin and elastatinal were tested for their eventual effect on the cytotoxicity induced by intact DT. These protease inhibitors were, respectively, capable of inhibiting a large number of eukaryotic proteases able to act through distinct catalytic mechanisms: serine proteinases (EC 3.4.21), cysteine proteinases (EC 3.4.22), aspartic proteinases (EC 3.4.23) and elastase enzyme. It should be noted that the most apparent difficulties in using protease inhibitors in tissue culture result from their toxicity and instability. Indeed proteolytic mechanisms are vital for the cell; therefore, there is only a narrow margin between an effective and a toxic dose. For reproducible results, it is essential to use freshly made inhibitor solutions. Since it was observed that the effect of the protease inhibitors depends on the age and density of the cell cultures (Schnebli and Burger, 1972), we tested cytotoxicity on freshly seeded cells at low density. The cytotoxicity of unnicked DT was tested in the presence of antipain (50 µg/ml), leupeptin (50 µg/ml), pepstatin (25 µg/ml), chymostatin (25 µg/ml) or elastatinal (50 µg/ml). These concentrations were not toxic, as established by microscopic examinations with an inverted microscope and 0.1%, w/v trypan blue exclusion. No significant morphological changes were observed in the cells treated with these inhibitors of proteases: cells were flatter than untreated cells, but they still overlapped and remained adherent. The effects of five protease inhibitors on the cytotoxicity of DT are shown in figure 2. Cultures of B4 in the presence of unnicked DT were virtually indistinguishable from inhibition of protein synthesis in the absence or presence of protease inhibitors ($ID_{50} = 7 \times 10^{-12}$ M). None of these protease inhibitors interfered with the cytotoxicity of DT. By using protease inhibitors, we uniformly failed to even partly inhibit the cytotoxicity of unnicked DT. This experiment strongly suggested that DT could be processed

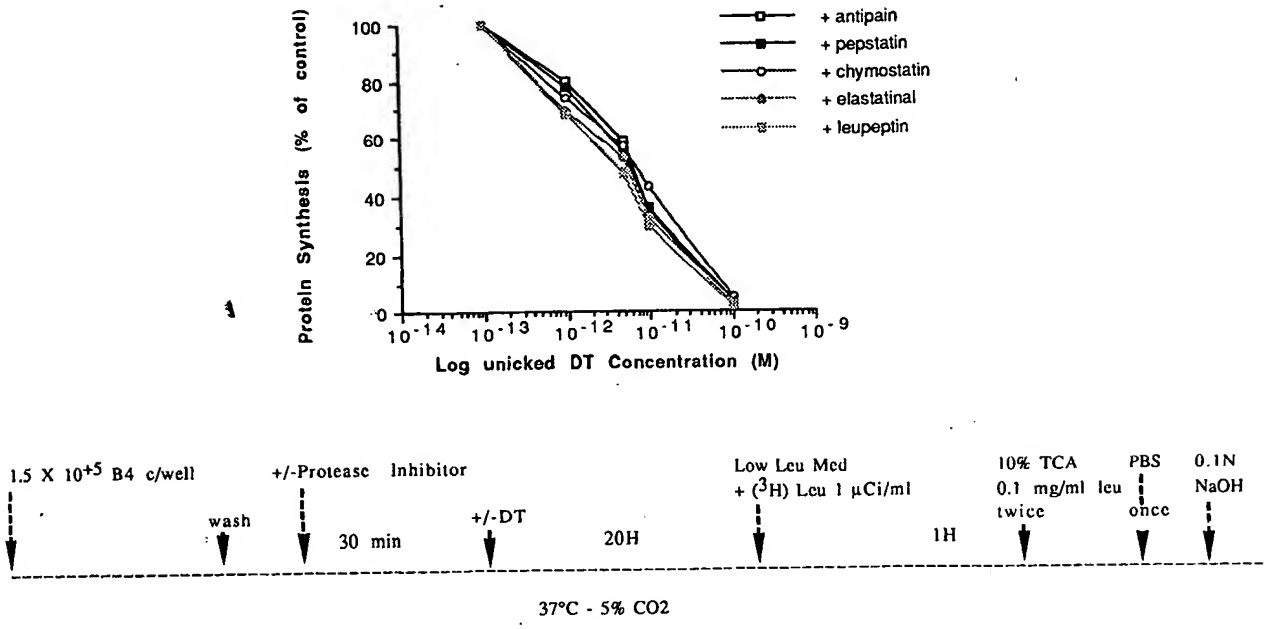

Fig. 2. Cytotoxicity of unnicked DT in the presence of inhibitors of cellular proteases.

Unnicked DTs were incubated with antipain (50 µg/ml), leupeptin (50 µg/ml), pepstatin (25 µg/ml), chymostatin (25 µg/ml) or elastatinal (50 µg/ml).

and thus activated via several different proteases to exert its intracellular toxic action.

To pursue the exploration of cell-associated protease involved in the processing of DT, we investigated the eventual implication of a secreted serine endopeptidase, the plasminogen activator urokinase (uPA) (EC 3.4.21.73). Indeed, in agreement with Cieplak and Eidels (1987), we observed (figure 3) that the *in vitro* digestion of DT with urokinase gave two fragments corresponding to 21,167 and 37,199 daltons (respectively, to A and B fragments). This result underlined the role of urokinase as a putative protein molecule involved in intoxication by DT. To ascertain whether the Vero cell line is a relatively good secretor of the proenzyme form of urokinase, we performed electrophoretic analysis of 10 µl serum-free Vero cell culture (conditioned culture medium) in polyacrylamide gels as described in "Materials and Methods", in the presence or absence of copolymerized plasminogen (15 µg/ml) as sequential substrates. This experiment, in agreement with Heussen and Dowdle (1980), exploited the fact that the catalytic activity of urokinase was reversibly inhibited by SDS in the electrophoresis gel. As can be seen in figure 4, we detected five sharp clear fibrinolysis bands. The enzymes released by cultured Vero cells showed three major bands of plasminogen-dependent proteolysis with a spectrum of molecular weight. The two most prominent bands appeared as a closely spaced enzyme doublet with molecular weights of approximately 60 and 58 kDa measured. These bands corresponded to the components of urokinase-type enzymes with a concentration > 1.5 IU/ml Vero cell harvest fluid assigned to the enzymes by interpolation from the standard urokinase. Our results demonstrated that uPA was secreted by Vero cells.

In an attempt to shed light on a specific and active role of urokinase in the *in vivo* cleavage of DT, we first used neutralizing antibodies directed against urokinase, i.e. goat anti-uPA IgG (human LMW), purchased from DiaMed-France S.A., Paris, France. We tested anti-urokinase antibodies

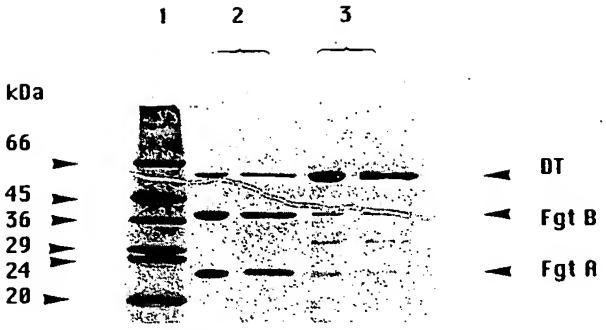

Fig. 3. *In vitro* cleavage of highly purified DT by urokinase.

Unnicked DTs (500 µg/ml) were incubated alone or with urokinase (3,750 IU/ml) in PBS at room temperature for 3 h; 6 µg of DT were denatured by boiling for 5 min in 150 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer pH 7.2 and analysed by SDS-PAGE in 11% resolving gels with 5% (v/v) of 2-mercaptoethanol. The gels were fixed and stained with Coomassie blue. Lane 1=molecular weights; lanes 2=unnicked DT treated with urokinase for 1 and 3 h, respectively; lanes 3=unnicked DT incubated with PBS for 1 and 3 h, respectively.

for their ability to antagonize cytotoxicity of unnicked DT in Vero cells. In this experiment, described in figure 5, we added a concentration of unnicked or nicked DT required to allow 50% inhibition of protein synthesis as a result of a 5-h exposure to toxin in the presence or absence of anti-urokinase antibodies. We measured cytotoxicity by assaying the rate of protein synthesis and the rate of DNA synthesis in cultured cells. Interestingly, partial inhibition of the cytotoxicity of unnicked DT was recorded in the presence of anti-urokinase antibodies (fig. 5). Furthermore, anti-urokinase antibodies blocked the cytotoxicity of unnicked DT in a dose-dependent fashion. We defined the inhibitory activity (I) according to the following relation: (I) = (Ci-Ct)/(Co-Ct) × 100, where Co is the radioactivity (cpm) incorporated by cells without DT and without anti-urokinase antibodies, Ct is the count incorporated by cells with DT and without anti-urokinase antibodies, and Ci is the count incorporated by cells with both DT and anti-urokinase antibodies. We recorded a maximal inhibitor activity of anti-urokinase antibodies equal to 66 units in the presence of unnicked DT, whereas inhibitor activity was less than 4 units in the presence of nicked DT under the assay conditions. In this experiment, anti-urokinase antibodies were effective inhibitors of unnicked DT. In contrast to unnicked DT, anti-urokinase antibodies had no effect on the cytotoxicity mediated by trypsin-treated DT. Thus, the protective effect of anti-urokinase antibodies was not due to indirect effects on the cells. These results demonstrated that urokinase is involved and is necessary for the cytotoxicity of DT.

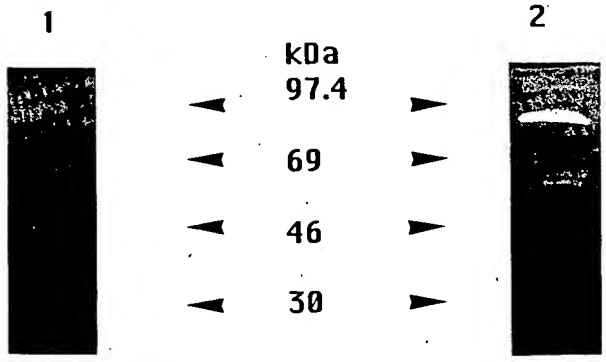

Fig. 4. Identification of plasminogen-dependent proteases in polyacrylamide containing gelatin and plasminogen.

Serum-free Vero cell harvest fluid (10 µl) in gels containing SDS-PAGE (11%), copolymerized gelatin (0.1%, w/v), without (lane 1) and with (lane 2) copolymerized plasminogen (15 µg/ml).

Taken together, these results confirmed the hypothesis advanced by Cieplak *et al.* (1987, 1988) of the possible participation of urokinase as an "activator" of DT in the intoxication process in cells such as Vero cells. Clearly, urokinase is a good candidate to efficiently promote *in vivo* activation of DT, since: (i) urokinase is widely secreted by a variety of normal tissues showing sensitivity to DT and, (ii) urokinase is surface-located (to its surface receptors, uPAR (Roldan *et al.*, 1990), with a very high affinity (0.2 nM), thus allowing a high local concentration of the plasminogen activator (PA) close to the cells and avoiding dilution in body fluids. Recently, Tsuneoka *et al.* (1993) established that a membrane-associated, calcium-dependent serine endoprotease with a subtilisin-like catalytic domain, furin (Molloy *et al.*, 1994), plays a substantial role in the activation of DT (Tsuneoka *et al.*, 1993). On the other hand, Gordon *et al.* (1995), in disagree-

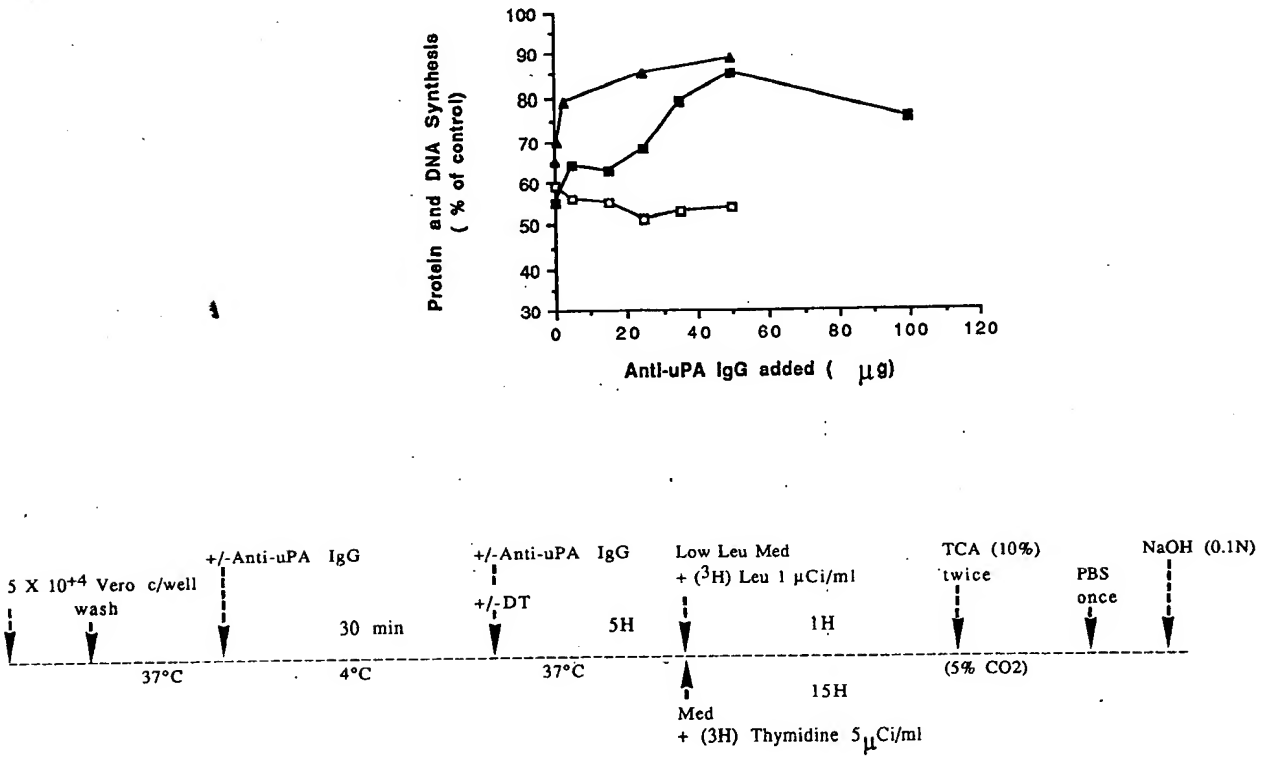

Fig. 5. Cytotoxicity of unnicked or nicked DT in the presence of anti-human low-MW uPA.

Vero cells were cultured with DT (5×10^{-12} M final) and increasing concentrations of anti-human low-MW uPA. Non-toxin-treated cells ranged from 9,500 to 11,000 cpm (^{3}H-leucine incorporation) and 16,500 to 17,000 cpm (^{3}H-thymidine incorporation). Protein synthesis in the presence of nicked DT (□) or unnicked DT (■). DNA synthesis in the presence of unnicked DT (▲).

ment with Tsuneoka et al. (1993), showed that the sensitivity of furin-deficient cells to DT was reduced only slightly from that of wild-type cells. These studies provide evidence for the contribution of at least one additional protease in the activation of DT. Furin is the major protease that contributes to the activation of DT and anthrax toxin PA (Klimpel *et al.*, 1992; Molloy *et al.*, 1992, 1994). Based on our experiments, we showed that urokinase contributed to the cytotoxicity of DT on Vero cells and thus was involved in the activation of DT. However, anti-urokinase antibodies failed to totally protect Vero cells, suggesting that urokinase was not sufficient for the full nicking of DT. Our results, taken together with the work cited, rule out assigning a functional role for the cleavage of DT to a unique protease, in contrast to the case of *Pseudomonas* exotoxin A (Fryling *et al.*, 1992). In view of these findings, it is tempting to speculate that the ultimate goal of DT activation, fully nicked, and therefore the sensitivity of a cell line to DT, would depend on the critical set of enzymatic equipment located in a structure to which toxin could be delivered and processed. The protease primarily responsible for cleavage of DT in this phenomenon might be different depending upon specific cell types and changing physiological situations. With respect to the relevance of the *in vivo* protease contribution, one may question the extent to which results obtained with the *in vitro* cultured cells can give insight into the functional status of enzymes *in vivo*. In this view, we would like to underline that quantitative and qualitative

differences exist between normal and malignant cells in terms of rates of proteinase synthesis and release. Some enzymes are selectively secreted by malignant cells but not by their normal counterparts, suggesting that the overexpression of the uPA is one of the features of a number of immortalized cell lines (Markus *et al.*, 1983). Therefore, it remains possible that *in vitro* conditions of cell lines could amplify the contribution of these enzymes during DT intoxication.

Acknowledgments

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Rôle de l'urokinase activatrice du plasminogène lors de la cytotoxicité *in vitro* de la toxine diphtérique

Une coupure de la toxine diphtérique (DT), dans une région riche en arginine au sein du premier pont disulfure, est indispensable pour le processus d'intoxication par la toxine. Nous montrons que la protéase requise pour une telle protéolyse est synthétisée et sécrétée par les cellules sensibles. De plus, des anticorps dirigés contre l'urokinase diminuent *in vitro* la cytotoxicité de la DT sur la lignée cellulaire Vero. Nos résultats démontrent que l'urokinase sécrétée par les cellules Vero est une des protéases cellulaires impliquées dans la protéolyse et l'activation de la toxine diphtérique.

Mots-clés : Toxine, *Corynebacterium diphtheriae*, Urokinase ; Activatrice du plasminogène, Cytotoxicité.

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Cloned Fragment of Diphtheria Toxin Linked to T Cell-specific Antibody Identifies Regions of B Chain Active in Cell Entry*

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Marco Colombatti‡§, Lawrence Greenfield¶, and Richard J. Youle‡

From the ‡Surgical Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892 and the ¶Cetus Corporation, Emeryville, California 94608

The role of discrete domains of diphtheria toxin (DT) B chain in cytosol entry and cytotoxicity was investigated by linking a monoclonal antibody recognizing the human T cell-specific antigen T3 (UCHT1) to diphtheria toxin (UCHT1-DT), DT A subunit (UCHT1-DTA), or to a genetically engineered form of DT (UCHT1-MspSA) lacking the C-terminal 17-kDa portion of the B subunit. The N-terminal 21-kDa region of DT B chain increased toxicity of UCHT1-DTA 100-fold (UCHT1-MspSA) while addition of the C-terminal 17-kDa region (UCHT1-DT) increased toxicity 100-fold more. The cytotoxicity was dependent upon antibody binding as demonstrated by blocking toxicity with excess UCHT1. The differences in toxicity between these reagents were not due to differences in ADP-ribosylation activity of DT A chain, binding activity of the antibody moiety, extent of DT nicking, or the cross-linking method, so we conclude that the large differences in toxicity were due to the presence of different B chain domains. The large increase in toxicity by the C-terminal region of DT B did not appear to be caused by DT receptor binding. The lysosomotropic agent NH_4Cl blocked the cytotoxic effect of DT, UCHT1-DT, and UCHT1-MspSA but not UCHT1-DTA.

Diphtheria toxin (DT[1]) is comprised of two disulfide-linked subunits; the 21,000-dalton A chain inhibits protein synthesis by catalyzing the ADP-ribosylation of elongation factor 2 (1) and the 37,000-dalton B chain binds cell surface receptors (2). The cell surface-binding domain and the phosphate-binding site (3) are located within the carboxyl-terminal 8-kDa cyanogen bromide peptide of the B chain (4). Close to the C terminus region of DT B chain are several hydrophobic domains (Fig. 1 in Miniprint and Ref. 5) that can insert into membranes at low pH and appear to be important for DT entry (6–9).

Antibodies directed against cell surface antigens have been linked to intact diphtheria toxin or its A subunit to selectively kill antigen-bearing target cells (10–12). Antibody toxin (immunotoxins) or ligand toxin conjugates containing only the DT A chain have relatively low cytotoxic activity (13, 14) while intact DT conjugates can be very potent (15, 16), but the two types of immunotoxin have never been compared in the same system in a sensitive species. Since the DT B chain appears to facilitate entry of DT A chain to the cytosol, it is possible that its presence in whole toxin conjugates renders them more potent although less specific. Efforts have been made to construct more potent and specific immunotoxins by separating the toxin B chain domains involved in cell binding from the domains involved in A chain entry (17–19).

Recombinant DNA technology offers a powerful approach to improve immunotoxin efficacy at the gene level. Greenfield *et al.* (20) have cloned portions of DT and created a modified toxin (MspSA) which contains the N-terminal hydrophobic region of DT but lacks the C-terminal 17-kDa region and contains a new C-terminal cysteine for ease of linking to antibodies.[2] This fragment, like CRM 45 (2), lacks the cell surface-binding site of DT but includes most of the hydrophobic region thought to facilitate membrane transport (Fig. 1 and Ref. 5).

We have examined the role of DT B chain domains in cell entry by comparing the activities of a T cell-specific monoclonal antibody, UCHT1, linked to DT A chain, MspSA, and intact DT containing the whole B chain. The N-terminal region of DT B chain increases antibody specific toxicity 100-fold, and the C-terminal region increases activity 100-fold more.

EXPERIMENTAL PROCEDURES AND RESULTS[3]

Effect of Immunotoxin Treatment on Jurkat Leukemia Cells—We compared the toxicity of a monoclonal antibody, UCHT1, linked to three forms of diphtheria toxin which varied in the length of the diphtheria toxin B subunit sequence included. Antigen (T3)-positive human leukemia cells (Jurkat) were incubated with the three immunotoxins, and the protein synthesis rate was determined. As seen in Fig. 2, UCHT1-DTA decreased the protein synthesis of treated cells to 50% of control at about 2×10^{-8} M; UCHT1-MspSA inhibited protein synthesis 50% at about 3×10^{-10} M, and UCHT1-DT showed the highest cytotoxic activity, reducing the protein synthesis to 50% of control cultures at 3×10^{-12}

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§ Dr. M. Colombatti is on leave of absence from the Istituto di Scienze Immunologiche, Università di Verona, Italy.

[1] The abbreviations used are: DT, diphtheria toxin; EF-2, elongation factor 2; Kd, kilodaltons; IT, immunotoxins; MBS, *m*-maleimidobenzoyl-N-hydroxysuccinimide ester; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)-propionate; DTT, dithiothreitol; DTA, diphtheria A chain.

[2] L. Greenfield, H. Dovey, and D. Nitecki, manuscript in preparation.

[3] Portions of this paper (including "Experimental Procedures," part of "Results," and Figs. 1, 4–7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-2526, cite the authors, and include a check or money order for $6.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

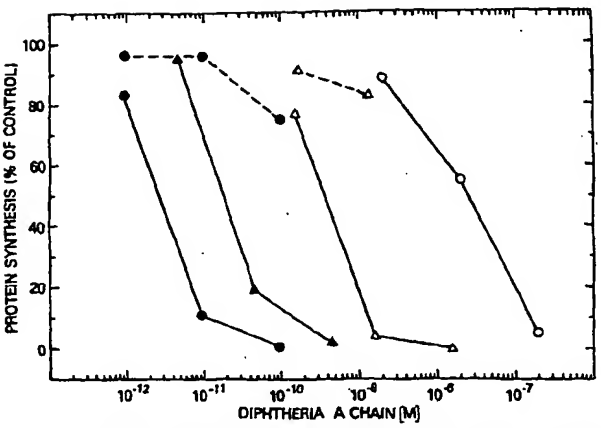

FIG. 2. **Effect of 23.5-h immunotoxin treatment on Jurkat target cells.** One hundred thousand Jurkat cells/well (100 μl) were cultured for 23 h at 37 °C in the presence of the various reagents or control solutions. Cells were then pulsed for 1 h with [^{14}C]leucine. ●—●, UCHT1-DT; △—△, UCHT1-MspSA; ○—○, UCHT1-DTA; ▲—▲, DT; ●--●, UCHT1-DT + UCHT1 (100 μg/ml); △—△, UCHT1-MspSA + UCHT1 (100 μg/ml).

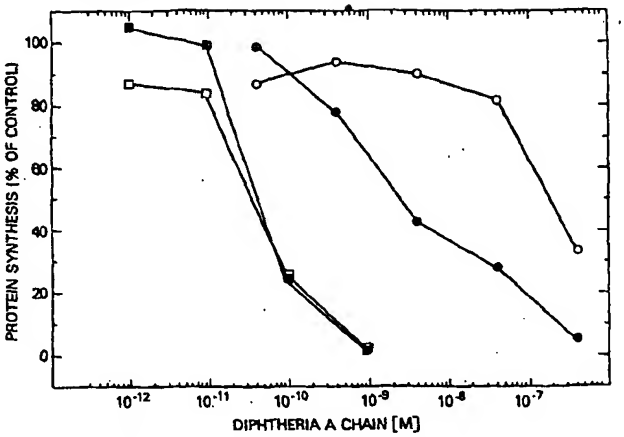

FIG. 3. **Effect of DT-binding site blockade on DT and UCHT1-DT cytotoxic activity.** One hundred thousand Jurkat cells were cultured for 3 h at 37 °C (100 μl) in the presence of the different reagents or control solutions. After this, cells were pulsed for 1 h with [^{14}C]leucine. ■—■, UCHT1-DT, □—□, UCHT1-DT + CRM 197 (10^{-5} M); ●—●, DT; ○—○, DT + CRM 197 (10^{-5} M).

M. UCHT1-DT was, therefore, about 100-fold more potent than UCHT1-MspSA and about 10,000-fold more potent than UCHT1-DTA in a 23.5-h assay. Free UCHT1 blocked toxicity of UCHT1-DT and UCHT1-MspSA about 100- and 10-fold, respectively (Fig. 2), but did not affect the toxicity of native DT. Assays performed at 3.5 h showed an almost identical relationship of the three immunotoxins (Fig. 4, Miniprint) although they only killed cells at higher concentrations.

The immunotoxin concentration was based on ADP-ribosylation activity; therefore, the toxins are exactly comparable in regard to A chain potency. The binding of immunotoxin was examined and is shown in the Miniprint not to account for the large differences in immunotoxin potency. The nature of the cross-link, both disulfide and thioether, is also shown in the Miniprint and revealed no differences in the above pattern of toxicity. Therefore, we conclude that the 100-fold higher toxicity of UCHT1-MspSA over UCHT1-DTA is due to the activity of the N-terminal 15-kDa region of DTB while the 100-fold increase in toxicity of UCHT1-DT over UCHT1-MspSA is due to the activity of the C-terminal region of the DT B chain.

Effect of DT-binding Site Blockade on UCHT1-DT Cytotoxic Activity—We have demonstrated that the toxicity of UCHT1-DT is antibody mediated and that the C terminus region, which contains the DT surface-binding activity, is needed for high potency. In the following experiments we examined whether the binding of antibody-linked DT to cell surface receptors is required as a second step in the process of cell intoxication by antibody-DT immunotoxins. Assays were, therefore, performed in the presence of a mutant DT (CRM 197 (21)) which binds DT receptors but is nontoxic due to a single amino acid substitution at position 52 of DT A chain which completely blocks ADP-ribosylation (22). As Fig. 3 shows, 10^{-5} M CRM 197 blocked DT toxicity 90-fold but did not affect the cytotoxic activity of UCHT1-DT. Thus, we could not show any effect on UCHT1-DT toxicity by blocking 99% of the surface DT receptors. Phytic acid, a competitive inhibitor of DT binding (23), blocked DT toxicity about 70-fold but had no significant effect on UCHT1-DT (data not shown).

DISCUSSION

Diphtheria toxin requires functions present on both of its disulfide-linked subunits for toxicity. The A chain inhibits protein synthesis when in the cytosol (24) while the B subunit binds cell surfaces (21, 25) and is thought to facilitate entry of the A subunit into the cytosol. The C-terminal 17-kDa region of DT B chain appears to partially or fully comprise the cell surface-binding site since 1) CRM mutants lacking this region (*i.e.* CRM 45) fail to bind cells (21) and 2) monoclonal antibodies which bind the C-terminal region block binding (26). Four hydrophobic domains exist in the DT B chain, three contained in the 21-kDa N terminus and one in the 17-kDa C terminus (5). At low pH, DT undergoes a conformational change which exposes hydrophobic domains and inserts into lipid bilayers (9). Eliminating low pH environments intracellularly blocks DT passage to the cytosol (8, 9, 27) while lowering extracellular pH to 4.5 circumvents this block implying that exposure of the hydrophobic domains is necessary for cell entry. Treatment of DT *in vitro* at pH 3.0–5.0 inactivates toxicity but not A chain enzymatic activity (9).

We have investigated the role of the DT B chain domains in cell entry as distinguished from cell binding by adding a new binding moiety to various size derivatives of DT. We found UCHT1 monoclonal antibody linked to diphtheria toxin A chain had low toxicity, but addition of the N terminus of DT B (as in UCHT1-MspSA) increased toxicity 100-fold. This is similar to work by Bacha *et al.* (18) who found that thyrotropin-releasing hormone linked to CRM45 was more toxic than thyrotropin-releasing hormone linked to the DT A chain analog, CRM 26. Presumably the three hydrophobic domains of MspSA and CRM 45 facilitate passage of DT out of intracellular vesicles. Interestingly, NH_4Cl blocked UCHT1-MspSA indicating that, like DT, it requires a low pH environment for membrane passage. UCHT1-DTA was not blocked by NH_4Cl, another indication that low pH results in the exposure of hydrophobic domains present on B chain but not on DT A chain.

Addition of the C-terminal 17-kDa region of DT B by linking intact DT to UCHT1 further increased toxicity more than 100-fold even though the binding was mediated solely by the antibody. What activity of this C-terminal region facilitates DT entry? We tested the possibility that cell surface DT receptors were involved. CRM 197 has no toxicity but binds DT receptors and blocks DT toxicity by competing for binding sites (21–24). Our data show that CRM 197 blocks

DT toxicity to Jurkat 100-fold but has no effect on UCHT1-DT. This indicates that the C terminus does not function solely by binding cell surface receptors; rather, this part of the molecule could be involved in binding of intracellular receptors or in membrane transport functions.

Intracellular binding of ricin receptors was proposed as the function of ricin B chain which facilitated immunotoxin toxicity (17). The results reported here for diphtheria toxin are consistent with the model proposed for ricin.

How hydrophobic domains insert into membranes is not known. Low pH inactivates DT by altering the B subunit (9). We used UCHT1-DT to determine whether this conformational inactivation was due to DT binding or entry functions. UCHT1-DT was inactivated by treatment at pH 4.0 even though cell binding by UCHT1 was maintained; therefore, the precise DT B subunit conformation appears to be necessary for membrane transport rather than binding. The sensitivity of UCHT1-MspSA to acid treatment revealed that the requirement for a proper conformation is not restricted to the C-terminal domains missing from MspSA, but also extends to other parts of DT B chain.

Antibody toxin conjugates are useful reagents for *in vitro* cell depletion. One goal is to use these reagents *in vivo* for cancer therapy. Antibodies linked to toxin A chains often have kinetics so slow as to be inactive *in vivo* while antibodies linked to intact toxins have too much nontarget cell toxicity for *in vivo* administration. Functions of toxin B chains which would accelerate activity of antibody toxin conjugates but not contribute to nontarget cell toxicity *in vivo* would be important to identify. The 21-kDa N terminus of DT B appears to greatly increase DTA-UCHT1 activity but makes little contribution to nontarget cell toxicity. Activities in the 17-kDa C terminus of DT B chain could be used to increase toxicity 100-fold more if they can be separated from cell surface-binding activities.

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SUPPLEMENTARY MATERIAL TO: "CLONED FRAGMENT OF DIPHTHERIA TOXIN LINKED TO T CELL SPECIFIC ANTIBODY IDENTIFIES REGIONS OF B CHAIN ACTIVE IN CELL ENTRY"

by

Marco Colombatti , Lawrence Greenfield and Richard J. Youle

EXPERIMENTAL PROCEDURES

Reagents

Diphtheria toxin (m.w 65Kd), 95% nicked, was purchased from Calbiochem; Diphtheria toxin, <10% nicked, was purchased from List Biologicals. UCHT1, an IgG1 which recogaizes the CD3 (p20) determinant was a generous gift from Unilever, Bristol, England. m-Maleimidobenzoyl N-hydroxysuccinimide ester (MBS), n-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and dithiothreitol (DTT) were from Pierce Chemical Co., Rockford, IL.

Diphtheria toxin A chain (21kDa) was purified from reduced and denatured DT according to the methods described by Chang and Collier (28) and Sandvig and Olsnes (9). EF-2 was purified from rat liver cells following a procedure described by Moldave (29). CRM 197 was purified by ion exchange on DEAE Sepharose.

MspSA

The MspSA toxin is a recombinant derivative of DT synthesized in E. Coli using the Msp restriction fragment of Corynebacterium diphtheriae genome; it consists of amino acids 1 through 382 of the native DT sequence (20) to which the nucleotide sequence encoding for the following aminoacid sequence has been added to the C-terminal end by conventional techniques: leu-pro-gly-thr-gly-ser-gly-prog-ser-gly-ser-gly-thr-cys (Fig. 1). Further details on recombinant DNA techniques used for MspSA construction and expression will be published elsewhere.[2] MspSA was purified by passage through a DEAE column equilibriated with 10mM Na phosphate pH 6.8 and eluted with a 0-300 mM Na Cl gradient. The eluted material was then loaded onto a NAD-agarose column in 10mM Na phosphate and affinity-purified by eluting the bound material with a 0-1.2 M Na Cl gradient. The concentrated eluate was then further purified by running through a Sephacryl S-200 sizing column. MspSA was obtained as a >95% pure protein of 45,000 dalton according to SDS-polyacrylamide gel electrophoresis. Its EF-2 ADP-ribosylating activity was equal to nicked DT.

Immunotoxins

Thioether and disulfide-linked immunotoxins (IT) were made by slight modification of previously published procedures (30, 31); DT and MspSA were linked to MBS by incubating with a 5 fold excess of MBS for 30 min at room temperature; for disulfide linkage, MspSA and DT A chain were reduced with 100 mM DTT for 2 hr followed by gel filtration and mixing with UCHT1 previously modified with a 7 fold molar excess of SPDP. Immunotoxins were purified by gel filtration on a TSK-3000 HPLC column. The immunotoxins ran as distinct peaks in front of unconjugated antibody. All immunotoxins were a mixture of conjugates of different stoichiometry but showed an average m.w. corresponding to 1:1-1:3 (antibody:toxin) molar conjugates and were separated from unconjugated antibody and toxin.

Protein Synthesis Assay

The *in vitro* adapted human leukemic T cell line Jurkat, maintained by serial passage in RPMI 1640 + 10% FCS was used throughout our study. Protein synthesis was assayed by incubating 10^5 cells in 80μl leucine free RPMI medium containing 2% FCS in 96 well microtiter plates; toxins, immunotoxins and control solutions were added in 10 μl to a final volume of 100 μl and incubated with cells for 3 hr or 23 hr. Twenty μl of medium containing 0.1 μCi of ^{14}C-leucine were then added for 60 min. Cells were then harvested onto glass fiber filters with a PHD cell harvester, washed with water, dried and counted. Incubation time is calculated as preincubation time plus one half the ^{14}C-leucine pulse time. All cytotoxicity assays were performed 2-4 times; data showed less than 10% variation between different experiments. Values represent the average of duplicate samples (less than 10% standard error). The results are expressed as a percentage of the incorporation in the mock-treated control cultures. The incorporation in the controls varied between 16,000 and 22,000 cpm.

EF-2 ADP-ribosylation assay

ADP-Ribosylation of EF-2 was assayed by a modification of a previously described method (32). Briefly, 50 μl of partially purified EF-2 were dispensed in a 96 well microtiter plate followed by 25 μl ^{32}P-Nicotinamide adenine dinucleotide (0.9 μCi, specific activity 707.1 Ci/mmol). DT, DT A chain, immunotoxins and control solutions were added in 25 μl and mixtures incubated for 15 min at room temperature. All reagents added were in 0.25M Tris-HCl buffer pH 8.2 containing 0.04 M DTT and 0.1 M EDTA. The reaction was stopped by filling the wells with 5% trichloroacetic acid, the precipitate harvested onto glass fiber filters, washed, dried and counted. The background of the assay was determined by replacement of toxins or immunotoxins with Tris-HCl buffer. Standard curves were established by assaying threefold dilutions of nicked DT at known concentrations. Concentration of toxins or immunotoxins were then determined based on DT standard curves, these concentrations were comparable to concentrations based on absorbance (O.D. 280 nm). UCHT1-DT used in this study was synthesized with 95% nicked DT. Extent of conjugation was determined by pre-incubating 100 μl of the immunotoxin solution with 50 μl packed goat anti-mouse Ig antiserum covalently coupled to Sepharose 4-B. After 30 min incubation at room temperature samples were centrifuged at 12,000 rpm for 2 min and the supernatant was assayed for EF-2 ADP-rybosylation as above.

Immunotoxin Binding Assay

Binding of UCHT1-DT, UCHT1-MspSA and UCHT1-DTA was estimated by flow cytofluorometry. One million cells in 100 μl medium + 10% FCS were incubated with 100 μl UCHT1 or immunotoxins for 30 min on ice. Cells were then washed and resuspended in 200 μl medium + FCS; 10 μl of fluoresceinated goat antimouse immunoglobulins (Ig) antiserum were then added and cells incubated for 30 min on ice. After this, cells were washed again and analyzed on a Coulterfluorescence activated cell sorter.

pH Treatment

DT, UCHT1-DT and UCHT1-MspSA (20 μg/ml, 4 μg/ml and 2 μg/ml respectively) in 10 mM Na acetate pH 4.0 or in PBS pH 7.0 were incubated at 37°C for 1 hr. After this treatment, the samples were neutralized and 10 μl of each solution were separately added to 90 μl culture medium containing 10^5 cells and a protein synthesis assay was performed as described above.

RESULTS

Role of Cross-linking Method

UCHT1-DT is a thioether conjugate, whereas UCHT1 is linked to MspSA via a disulfide bridge. The different cytotoxic potency of UCHT1-DT and UCHT1-MspSA might be explained by the different

cross-linking methods used for the conjugates synthesis. The thioether linkage used for UCHT1-DT conjugation results in a modification of amino groups of DT; conversely, the disulfide bond linking antibody and MspSA does not require chemical modifications of the toxin. A thioether cross-linked UCHT1-MspSA immunotoxin was synthesized by random derivatization and assayed in a protein synthesis inhibition assay. As seen in Fig. 5, miniprint, this immunotoxin only reduced protein synthesis 24% at the highest concentration (10^{-7}M). The higher potency of UCHT1-DT is therefore not due to the method of crosslinking. Comparison of UCHT1-DT conjugates synthesized with nicked or unnicked toxin revealed that the immunotoxins made with unnicked DT was about 10 fold more toxic(6).The difference in cytotoxicity between UCHT1-DT (unnicked) and UCHT1-MspSA (unnicked) was thus maintained or even greater and therefore nicking does not account for the difference in potency between the two immunotoxins.

Native DT reduced protein synthesis in Jurkat cells to 50% of control at 2.5×10^{-9}M in a 3.5 hr assay (Fig. 3) while MspSA alone was not toxic for Jurkat cells at concentrations as high as 6×10^{-6}M (Fig. 5, miniprint). Therefore the toxicity differential between target and non-target cells may even be greater for UCHT1-MspSA than UCHT1-DT.

Binding of Immunotoxins to Jurkat Cells

Differences in the ability to bind the surface of target cells might explain the lower cytotoxic properties of UCHT1-DTA and UCHT1-MspSA as compared to UCHT1-DT. To investigate this, Jurkat cells were treated with various concentrations of UCHT1 monoclonal antibody alone, with UCHT1-DT, UCHT1-DTA, or UCHT1-MspSA for 1 hr on ice. After washing, bound monoclonal antibody and immunotoxins were revealed by incubating the cells with a fluorescent goat anti-mouse Ig antiserum. The mean fluorescence intensity (MFI) expressed by the cells was then determined by flow cytofluorometry. As illustrated in Fig. 6, UCHT1-DT, UCHT1-MspSA, and UCHT1-DTA had comparable binding activity. In a replicate experiment a full binding curve of UCHT1-MspSA fell within 2 fold of the UCHT1 binding curve. MspSA lacks the cell surface binding domain, therefore binding of UCHT1-MspSA to the cell surface can only occur via UCHT1 monoclonal antibody. UCHT1-DT does not bind cells via the DT binding site since at the concentration used in the binding assay, UCHT1-DT toxicity can be totally blocked by an excess UCHT1 monoclonal antibody and is therefore mediated only by the antibody binding the target antigen. The binding of UCHT1-DTA to target cells was also demonstrated by the ability of UCHT1-DTA at 5×10^{-7}M to competitively block the cytotoxic effect of UCHT1-DT in a 3.5 hr protein inhibition assay (Fig. 4b, miniprint). The binding of all three immunotoxins was therefore comparable and could not explain the differences in toxicity.

Immunodepletion of Immunotoxin ADP-Ribosylation Activity

We conducted one further control to demonstrate that the concentration of UCHT1-MspSA and UCHT1-DTA as determined by ADP-ribosylation was not artifactually low due to free MspSA or DT-A chain present in the sample and not linked to UCHT1. DT, UCHT1-DT, UCHT1-MspSA and UCHT1-DTA were incubated with a goat anti-mouse Ig serum covalently bound to Sepharose 4-B beads, centrifuged and the EF-2 ADP-ribosylating activity remaining in the supernatant was assayed as described under Experimental Procedures. Sixty-four percent of the EF-2 ADP-ribosylating activity of UCHT1-DT preparation was precipitated and was therefore antibody-bound toxin; 99% of the EF-2 ADP-ribosylating activity found in the UCHT1- MspSA preparation was bound to UCHT1 antibody and was precipitated by insolubilized goat anti-mouse Ig antiserum and 73% of the ADP-ribosylating activity was immunodepleted from UCHT1-DTA, while only 7.5% of the EF-2 ADP-ribosylating activity could be precipitated from a control sample containing DT alone. The concentrations of UCHT1-MspSA and UCHT1-DTA were therefore within 20% of the true value. The UCHT1-DT may have been slightly more toxic than shown but the overall relative toxicities of the three reagents remains valid.

Effect of Ammonium Chloride on Immunotoxin Activity

DT toxicity can be blocked by lysosomotropic amines that neutralize lysosomal pH and probably interfere with DT entry by preventing the exposure of hydrophobic domains needed for transmembrane passage (6-9). We therefore investigated whether ammonium chloride could affect the cytotoxic activity of DT or MspSA linked to UCHT1 antibody. As Fig. 4a miniprint, illustrates, the presence of 10 mM NH_4Cl during the 3.5 hr of the assay totally blocked UCHT1-DT and UCHT1-MspSA cytotoxicity. Under the same conditions the toxicity of DT for Jurkat cells was also completely abolished (Fig. 5, miniprint). Thus UCHT1-MspSA requires a low pH environment for entry like native DT. UCHT1-DTA was not blocked by the presence of 10mM NH_4Cl during a 23.5 hr assay but was potentiated about 2 fold. Fifty percent inhibition of protein synthesis was attained at 3×10^{-9} M in the presence and at 6×10^{-9} M in the absence of ammonium chloride. Toxicity of DTA was not affected by the presence of NH_4Cl during a 23.5 hr assay (data not shown). These data suggest that only protein domains in the DT B chain mediated entry need a low pH.

Effect of pH on UCHT1-DT Cytotoxic Activity

Treatment of DT at low pH induces an irreversible conformational change of the toxin molecule that results in a reduction of its toxicity (9). To determine if the conformation of the hydrophobic domains necessary for toxin entry is required for immunotoxin activity we treated UCHT1-DT, UCHT1-MspSA and DT at pH 4.0 for 1 hr at 37°C and after neutralization assayed their cytotoxic effect in a 3.5 hr protein synthesis inhibition assay. As Fig. 7 miniprint, illustrates, 50% inhibition of protein synthesis was observed at 300 ng/ml (5×10^{-9}M) for acid-treated DT, while a control sample treated at pH 7.0 showed a 50% inhibition at 60 ng/ml (10^{-10}M). Protein synthesis was decreased to 30% of control at about 30 ng/ml (10^{-10}M) by untreated UCHT1-DT and at about 150 ng/ml (5×10^{-10}M) by acid-treated UCHT1-DT (Fig. 7, miniprint). UCHT1-MspSA cytotoxicity was also similarly affected by low pH treatment: 78% protein synthesis inhibition was observed at about 40 ng/ml (1.5×10^{-9}M) for control UCHT1-MspSA as opposed to 200 ng/ml (8.2×10^{-9}M) for UCHT1-MspSA pre-treated at pH 4.0 (Fig. 7, miniprint). UCHT1 antibody binding was not affected by pH 4.0 treatment (data not shown).

Treatment at pH 4.0 reduced the cytotoxic activity of DT, UCHT1-DT and UCHT1-MspSA about 4 to 5 fold demonstrating that a specific conformation of DT is needed not only for cell surface binding but also for entry to the cytosol. The effect of low pH on UCHT1-MspSA indicates that acid sensitive regions exist in the N-terminal portion of B chain.

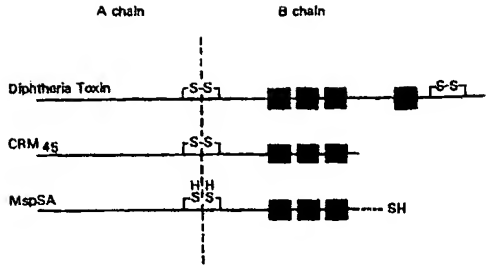

Fig. 1.

Structure of diphtheria toxin and related polypeptide molecules. Disulfide bonds are represented by the symbol -S-S-; black boxes represent hydrophobic sequences according to Ref. 5.

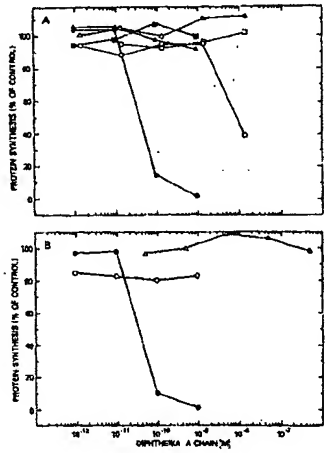

Fig. 4.

Effect of antibody-toxin conjugates on Jurkat target cells. One hundred thousand cells/well (100 µl) were cultured for 3 hr at 37°C in the presence of the various reagents or control solutions. Cells were then pulsed for 1 hr with ^{14}C-leucine, harvested and counted.

a) ●—● UCHT1-DT, ▲—▲ UCHT1-DT + UCHT1 $7X10^{-7}$M, ■—■ UCHT1-DT+ NH_4Cl 10mM ; o—o UCHT1-MspSA, △—△ UCHT1-MspSA + UCHT1 $7X10^{-7}$M, □—□UCHT1-MspSA + NH_4Cl 10 mM.

b) ●—● UCHT1-DT, Δ—Δ UCHT1-DTA, o—o UCHT1-DT + UCHT1-DTA $5X10^{-7}$M.

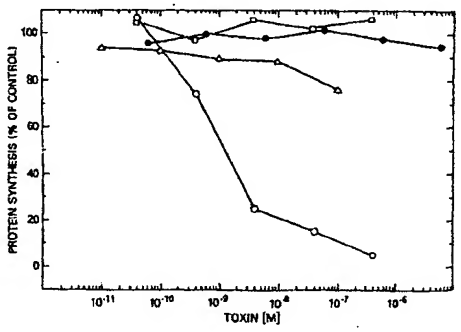

Fig. 5.

Effect of DT, MspSA and thioether-linked UCHT1-MspSA on Jurkat target cells. One hundred thousand Jurkat cells/well (100 µl) were cultured for 3 hr at 37°C in the presence or absence of DT, MspSA or UCHT1-MspSA. Cells were then pulsed for 1 hr with ^{14}C-leucine. o—o DT, □—□DT + NH_4Cl 10mM, ●—● MspSA, △—△thioether-linked UCHT1-MspSA.

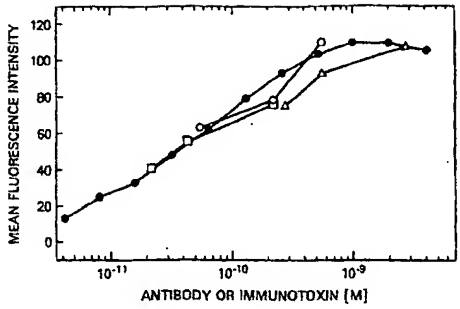

Fig. 6.

One million Jurkat cells were incubated with various dilutions of UCHT1 or IT for 30 min on ice. The cells were then washed and incubated for 30 min on ice with an anti-mouse Ig fluoresceinated antiserum. After washings, cell-bound fluorescence was analyzed by flow cytofluorometry. Cells treated with the second step reagent alone represented the background of the assay (subtracted). □—□ UCHT1-DT, Δ—Δ UCHT1-MspSA, o—o UCHT1-DTA, ●—● UCHT1.

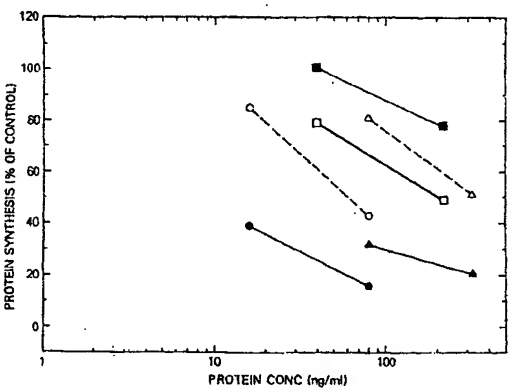

Fig. 7.

Effect of low pH pre-treatment on the cytotoxic activity of DT, UCHT1-DT and UCHT1-MspSA. DT, UCHT1-DT and UCHT1 -MspSA were exposed to pH 4.0 in Na acetate buffer for 1 hr at 37° C. Control samples were mock-treated at pH7.0. After neutralization, the cytotoxic activity of DT, UCHT1-DT and UCHT1-MspSA was assayed in a protein synthesis inhibition assay by incubating 10^5 Jurkat cells at 37° C (100 µl) for 3 hr with pH-treated or mock-treated DT, UCHT1-DT or UCHT1-MspSA. After this, cells were pulsed with ^{14}C-leucine. o---o DT, Δ---Δ DT pre-treated at pH 4.0; ●—● UCHT1-DT, ▲—▲ UCHT1-DT pre-treated at pH 4.0, □—□ UCHT1-MspSA, ■—■ UCHT1-MspSA pre-treated at pH 4.0.

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The Role of the Diphtheria Toxin Receptor in Cytosol Translocation*

(Received for publication, August 10, 1987)

Virginia Gray Johnson‡, Debra Wilson‡, Larry Greenfield§, and Richard J. Youle‡

From the ‡Surgical Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892 and the §Department of Microbial Genetics, Cetus Corporation, Emeryville, California 94608

The role of the receptor in the transport of diphtheria toxin (DT) to the cytosol was examined. A point-mutant form of DT, CRM 107 (CRM represents cross-reacting material), that has an 8,000-fold lower affinity for the DT receptor than native toxin was conjugated to transferrin and monoclonal antibodies specific for the cell-surface receptors T3 and Thy1. Conjugating the binding site-inactivated CRM 107 to new binding moieties reconstituted full toxicity, indistinguishable from native DT linked to the same ligand, indicating that the entry activity of the DT B chain can be fully separated from the receptor binding function. Like DT, the toxin conjugates exhibited a dose-dependent lag period before first-order inactivation of protein synthesis. Inactivation of the binding site of the toxin portion of the conjugate was found to have no effect on the kinetics of protein synthesis inactivation. The receptor used by the toxin determined the length of the lag period relative to the killing rate. Comparing the potency of CRM 107 conjugates with native DT, standardized for receptor occupancy, shows that new receptors can be as or more efficient than the DT receptor in transporting DT to the cytosol. The transferrin-CRM 107 conjugate, unlike native DT, was highly toxic to murine cells. All the data presented are consistent with a model that the DT receptor, other than initiating rapid internalization of the toxin to low pH compartments, is unnecessary for transport of the toxin to the cytosol and that membrane translocation activity is expressed by the DT B subunit independent of the receptor-binding site.

Diphtheria toxin (DT)[1] is composed of two disulfide-linked subunits, both of which are involved in the intoxication process (1). The A subunit catalyzes ADP-ribosylation of elongation factor 2 (EF-2), thereby stopping protein synthesis and killing the cell. The B subunit has two functions: binding to cell-surface receptors and translocation of the A subunit into the cytosol.

The existence of specific cell-surface receptors for DT was first demonstrated by Ittelson and Gill (2) and quantitatively studied by Middlebrook *et al.* (3). Recent evidence indicates that the DT receptor is an anion antiporter (4–8). This antiporter also appears to play a specific role in transport of the toxin to the cytosol because: 1) the presence of permeant anions is required for toxin entry (8); 2) binding and insertion of DT inhibit the anion antiporter (6); and 3) inhibitors of anion transport such as the disulfonated stilbene derivative SITS inhibit the binding and translocation of DT (4, 7).

Four hydrophobic domains are present in the B chain and appear to play an important role in the translocation of the toxin molecule to the cytosol (9, 10). Exposure to low pH is also necessary for DT entry. Low pH causes a conformational change in DT that exposes hydrophobic domains (11) and induces the insertion of the toxin into lipid bilayers (12).

We have shown previously that the binding function can be separated from the translocation function of DT (13). Two point mutations identified in CRM 107 at positions 390 and 525 reduce toxin binding 8,000-fold and reduce toxicity 10,000-fold. However, conjugation of CRM 107 to a new binding moiety, the monoclonal antibody to the T-cell antigen receptor, UCHT1, reconstituted full toxicity to the mutant. This immunotoxin exhibited full toxicity to antigen-positive target cells, indistinguishable from that of the native toxin linked to the same antibody, without the nontarget cell toxicity. The result was therefore an immunotoxin with a 10,000-fold increase in toxicity over native CRM 107.

This report examines the role of the receptor in the DT intoxication process. Since CRM 107 retains full DT entry activity, CRM 107 conjugated to various new receptor-binding moieties allows one to define the role that the receptors play in entry to the cytosol. DT entry via the DT receptor, the Thy1 antigen, the T3 subunit of the T-cell receptor, and the transferrin receptor is examined. All of the results are consistent with a model that the DT receptor is not required for translocation to the cytosol other than to mediate rapid internalization to low pH compartments. The B chain of DT appears to contain all the membrane translocation activity, and this activity is distinct and separable from the receptor-binding site on the toxin B subunit.

MATERIALS AND METHODS

Reagents—DT was purchased from List Biologicals (Campbell, CA). UCHT1, an IgG1 which recognizes the CD3 (p20) determinant, was from Unilever (Bristol, England). The rat anti-Thy1 monoclonal antibody (3A7) and the Jurkat cell line transfected with the murine Thy1.2 gene were a generous gift from Dr. Ethan Shevach (National Institute of Allergy and Infectious Diseases). Dithiothreitol, MBS, and 2-Iminothiolane were purchased from Pierce Chemical Co. Human transferrin and SITS were from Sigma. All cell culture supplies were from GIBCO.

CRM 107, developed by Laird and Groman (14), was purified by clarifying *Corynebacterium diphtheria* cultures by centrifugation. The supernatant was made 65% with ammonium sulfate, incubated at 4 °C overnight, and centrifuged. The pellet was resuspended in 50 mM Tris buffer, pH 8.0, containing 0.1 mM EDTA and dialyzed overnight at 4 °C against 50 mM Tris buffer, pH 8.0, containing 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. The sample was applied to a DEAE-Sepharose Fast Flow (Pharmacia LKB Biotechnology Inc.) column equilibrated in the same buffer as the dialy-

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[1] The abbreviations used are: DT, diphtheria toxin; CRM, cross-reacting material; SITS, 4-acetamido-4-isothiocyanostilbene-2,2′-disulfonic acid; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; EF-2, elongation factor 2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

sate. The CRM 107 was eluted by a linear gradient of NaCl up to 300 mM in the same buffer.

Cell Lines—WEHI-7 and EL-4 (murine T-cell lymphoma-derived cells), Jurkat and CEM (human leukemia T-cell lines), and K562 (a human erythroleukemia-derived cell line) were all grown in RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10 μg/ml gentamycin. Vero, a cell line derived from monkey kidney, and SNB 75, a glioblastoma-derived cell line, were maintained in Dulbecco's modified Eagle's medium containing the medium supplements described above. Jurkat cells transfected with the murine Thy1.2 gene (15) were grown in Dulbecco's modified Eagle's medium with the above supplements plus 5×10^{-5} M 2-mercaptoethanol, 1 μg/ml mycophenolic acid·25 mg/ml xanthine, and 15 μg/ml hypoxanthine. All cells were grown at 37 °C in 5% CO_2 in a humidified atmosphere.

Immunotoxins—UCHT1-DT and UCHT1-CRM 107 conjugates were made with a thioether linkage as previously described (16). The rat anti-Thy1 monoclonal antibody, 3A7 (17), was conjugated with DT according to this same protocol.

Conjugation of transferrin with DT or CRM 107 was performed by a modification of the antibody coupling procedure. Before conjugation, transferrin was loaded with iron according to the method of Shindelman *et al.* (18). 2-Iminothiolane was dissolved in 0.8 M boric acid, pH 8.5, and incubated with transferrin in an 8:1 molar ratio. After 1 h at room temperature, the modified transferrin was separated from 2-iminothiolane by gel filtration on a Sephadex G-25 column equilibrated with phosphate-buffered saline. MBS, dissolved in dimethylformamide, was added in 5-fold molar excess to the toxin. The mixture was incubated for 30 min at room temperature followed by chromatographic separation on Sephadex G-25. The MBS-conjugated toxin was mixed with thiolated transferrin in a 1:1.3 molar ratio and incubated for 3 h at room temperature, and immunotoxins were purified by gel filtration on a TSK-3000 high pressure liquid chromatography column. One-minute fractions were collected, and individual fractions were tested for toxicity using a protein synthesis inhibition assay. The A chain activity of the conjugate peak was quantified using the EF-2 ADP-ribosylation assay. This immunotoxin peak was used for all further experiments.

EF-2 ADP-ribosylation Assay—EF-2 was purified from rat liver cells following a procedure described previously (19). ADP-ribosylation was carried out in 80-μl reaction mixtures containing 40 μl of 0.01 M Tris-HCl buffer with 1.0 M dithiothreitol, pH 8.0, 20 μl of EF-2, and 10 μl of toxin sample. The reaction was initiated by the addition of 10 μl of [^{32}P]nicotinamide adenine dinucleotide (1.2 μCi, specific activity = 277 Ci/mmol, adjusted to 18 μM with cold nicotinamide adenine dinucleotide). Reaction mixtures were incubated at room temperature for 20 min and the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. The precipitate was washed once with 10% trichloroacetic acid solubilized in 0.1 M NaOH, and counted.

ADP-ribosylation activities of unknown samples were compared to values obtained from DT standards consisting of known concentrations of DT (values based on Lowry protein determination (20) using bovine serum albumin as a standard). The background of the assay was determined by replacement of the toxin with Tris-HCl buffer.

Protein Synthesis Assay—Inhibition of protein synthesis was used to assay the cytotoxic effects of DT, CRM 107, and the toxin conjugates. Cells growing in suspension cultures such as Jurkat, K562, EL-4, and WEHI-7 were washed once with leucine-free RPMI 1640 medium, without fetal calf serum, containing 17 mM HEPES, 10 μg/ml gentamycin, resuspended in this medium, and dispensed into 96-well plates at a density of 10^5 cells/100 μl/well. Adherent cells were trypsinized, washed in their regular growth medium, and dispersed in this medium into 96-well plates at a density of 5×10^4 cells/well. Cells were allowed to reattach and grow in the 96-well plate for 24 h. Before the protein synthesis assay was started, the cells were washed twice with the leucine-free medium described above, and the wells were refilled to a final volume of 100 μl. Toxin conjugates, toxins alone, or control solutions were added in 11-μl volumes, and the cells were incubated at 37 °C for a specific amount of time. Phosphate-buffered saline containing 0.1 μCi of [^{14}C]leucine (20 μl) was then added for 1 h, and the cells were harvested onto glass fiber filters using a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA), washed with water, dried, and counted. The results were expressed as a percentage of ^{14}C incorporation in the mock-treated control cultures. All cytotoxicity assays were performed two to five times. Values given represent the average of duplicate or triplicate samples with less than 10% standard error.

RESULTS

Reactivity of Transferrin-Toxin Conjugates on Various Cell Lines—We examined the activity of DT and CRM 107 linked to transferrin. Transferrin receptors are found in higher numbers in proliferating cells including tumor cells; (21–23) and because of this, antibodies to the transferrin receptor have been used in animal models for cancer therapy (24). The toxicity of CRM 107, DT, transferrin-CRM 107, and transferrin-DT conjugates to the human leukemia cell line Jurkat, the human erythroblastoma cell line K562, and the human glioblastoma-derived cell line SNB 75 were compared (Table I). Although CRM 107 is 10,000-fold less toxic than DT, transferrin-CRM 107 is equally toxic as transferrin-DT, showing that the new binding domain fully reconstituted the CRM 107 toxicity. Binding of the toxin conjugates is transferrin-mediated, as shown by the fact that free transferrin inhibits cytotoxicity (Table II). These results show that separation of DT binding and translocation noted with UCHT1 (13) occur via another cell-surface receptor and on a variety of different cell types.

The receptor occupancy at the IC_{50} concentrations for transferrin-CRM 107 on K562 cells and DT on Vero cells was calculated. Based on known receptor numbers and K_a values for transferrin on K562 (25) and DT on Vero cells (3), 3500 DT molecules on Vero cells were required to reduce protein synthesis to the same extent as 130 transferrin-CRM 107 molecules on K562 cells. Transferrin-CRM 107 is therefore 30-fold more toxic to K562 cells than is DT to Vero cells based on receptor occupancy.

Effects of Alternative Receptors on the Kinetics of Intoxication—The kinetics of protein synthesis inhibition by toxins binding three different receptors on the same cell were ex-

TABLE I

IC_{50} of DT and transferrin-toxin conjugates

	Jurkat	K562	SNB 75
	M	*M*	*M*
Tfn[a]-CRM 107	2×10^{-12}[b]	1.6×10^{-12}	1.7×10^{-11}
Tfn-DT	4×10^{-12}	9×10^{-13}	1.3×10^{-11}
CRM 107	5.4×10^{-7}	ND	2.5×10^{-8}
DT	6.1×10^{-11}	3.4×10^{-10}	2×10^{-12}

[a] Tfn, transferrin; ND, not determined.

[b] Concentrations shown are those that inhibit protein synthesis by 50% of control values. Jurkat cells were incubated with toxins for 16 h, followed by a 1-h pulse with [^{14}C]Leu. K562 and SNB 75 were incubated for 24 h, followed by a 1-h pulse.

TABLE II

Inhibition of toxicity by free UCHT1 or transferrin

Toxin	Cell line	Protein synthesis
		% control
UCHT1-CRM 107 (4×10^{-10} M)	Jurkat	3.9
+Free UCHT1 (100 μg/ml)	Jurkat	94.5
Tfn[a]-CRM 107 (3.6×10^{-10} M)	K562	0.7
+Free Tfn (300 μg/ml)	K562	100.3
Tfn-DT (2.3×10^{-10} M)	K562	2.4
+Free Tfn (300 μg/ml)	K562	110.3
Tfn-CRM 107 (7.5×10^{-9} M)	Vero	40.0
+Free Tfn (500 μg/ml)	Vero	70.0
Tfn-DT (6.5×10^{-9} M)	Vero	20.0
+Free Tfn (500 μg/ml)	Vero	30.0
DT (1×10^{-9} M)	Vero	6.9
+Free Tfn (500 μg/ml)	Vero	6.8
Tfn-DT (1.3×10^{-8} M)	Vero	4.9
+Free CRM 197 (65 μg/ml)	Vero	5.2
DT (1×10^{-8} M)	Vero	15.9
+Free CRM 197 (65 μg/ml)	Vero	84.1

[a] Tfn, transferrin.

amined. The inhibition of Jurkat cell protein synthesis produced by DT, UCHT1-CRM 107, and transferrin-CRM 107 was followed as a function of time, at different toxin concentrations. All three toxins exhibited a dose-dependent lag time before first-order inactivation of protein synthesis began. Concentrations of DT, UCHT1-CRM 107, and transferrin-CRM 107 that produced the same rate of protein synthesis inactivation were compared (Fig. 1*A*). The lag time before an identical rate of protein synthesis inhibition began varied among the three toxins. The transferrin receptor-mediated toxin had a lag time of 3 h. This was approximately twice that of the T3-mediated toxin (1.6-h lag), which in turn was approximately twice that of native DT (0.8-h lag). This shows that the receptor plays a role in determining the length of lag time before a given rate of protein synthesis inactivation begins.

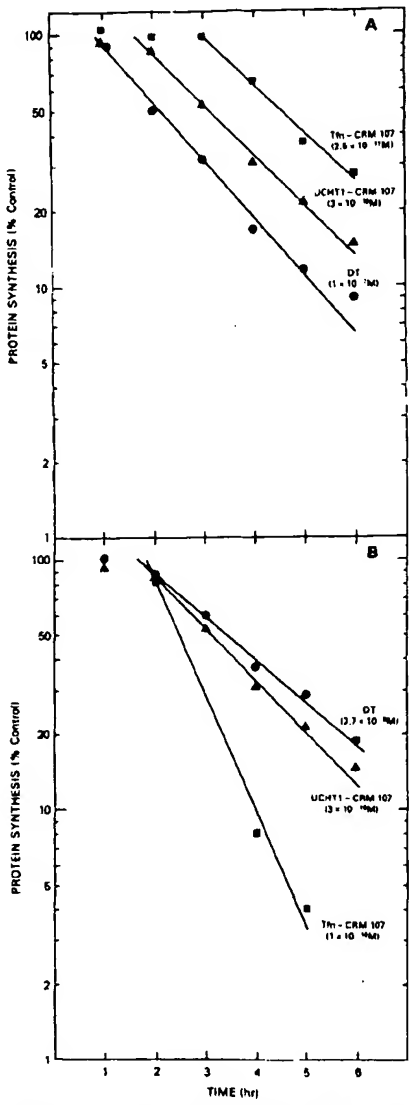

FIG. 1. **Effects of alternative receptors on the kinetics of intoxication.** Jurkat cells were incubated with DT, transferrin (*Tfn*)-CRM 107, or UCHT1-CRM 107 for the indicated times, followed by a 30-min pulse with [^{14}C]leucine. Cells were harvested, and ^{14}C incorporation was calculated as a percentage of mock-treated control cultures. *A*, concentrations of DT, UCHT1-CRM 107, and transferrin-CRM 107 producing the same rate of protein synthesis inactivation were compared. DT (1×10^{-7} M), UCHT1-CRM 107 (3×10^{-10} M), Tfn-CRM 107 (2.5×10^{-11} M). *B*, concentrations of DT, UCHT1-CRM 107, and transferrin-CRM 107 producing the same lag time were compared DT (3.7×10^{-9} M), UCHT1-CRM 107 (3×10^{-10} M), Tfn-CRM 107 (1×10^{-10} M).

When DT and the two CRM 107 conjugates were compared at concentrations producing the same lag time, the rates of intoxication varied greatly (Fig. 1*B*). Measured in logs/hour, transferrin-CRM 107 killed at double the rate of UCHT1-CRM 107 and approximately three times the rate of native DT after an identical lag time. DT assayed on Vero showed the same lag time and the same rate of protein synthesis inhibition as observed with Jurkat cells, differing only in the concentration required to reach this effect (26). The transferrin conjugates exhibited the longest dose-dependent lag period; but at the end of this lag they killed the most rapidly. Our results show that the ratio of lag time to first-order inactivation rate constant varies greatly depending upon the receptor used by DT. The receptor therefore defines the kinetics of cell killing.

Raso and Basala (27) conjugated transferrin with ricin A chain and measured the kinetics of intoxication. Using CEM cells, they found that the transferrin-ricin A chain conjugate (1×10^{-9} M) required 35 h to kill 50% of the cells. In contrast, we observed that the identical concentration of transferrin-CRM 107 required only 1.8 h to kill 50% of CEM cells (data not shown). This demonstrates that the B chain entry function of DT increased the killing rate 20-fold over ricin A chain. The B chain of ricin has been shown to accelerate killing rate only 5-fold (28).

Table III shows a comparison of the killing rates of the toxins and conjugates. The rate constant per bound immunotoxin ($K \times 5 \times 10^5/B$) is calculated to allow an accurate comparison of the killing mediated by different receptors on a variety of different cell types. It should be noted that the n and K_a values used in these calculations were taken from the literature and assumed to be identical both for the ligand alone and for the immunotoxin.

It was previously shown that UCHT1-DT was 10 times faster than UCHT1-ricin or T101-ricin (16). Transferrin-DT and transferrin-CRM 107, assayed on the same cell line (Jurkat), have killing rates approximately three times higher than UCHT1-DT. This demonstrates that the receptor plays an important role in the killing rate of the toxin. The native DT receptor is no more efficient than the transferrin receptor at mediating DT entry; and in some cell lines such as K562, the transferrin receptor is more efficient at mediating DT toxicity than is the DT receptor in Vero cells (Table III).

The kinetics of unconjugated CRM 107 on Vero cells was slower than native DT. However, since the concentration of CRM 107 required to give comparable number of toxin molecules bound was 4 logs higher than the concentration of DT, this difference may be due to a small error in the calculation of the amount bound.

UCHT1, conjugated to DT or to CRM 107, had a comparable killing rate (Table III). Similarly, the killing rates of DT or CRM 107 conjugated with transferrin were identical (Table III). Therefore, altering the binding site of the toxin portion of the conjugates had little or no effect on the kinetics of intoxication.

Role of the Anion Antiporter in Toxin Conjugate Translocation—Sandvig and co-workers (4–8) have reported that a potent inhibitor of the anion antiporter, SITS, inhibits DT binding and toxicity in Vero cells. These authors propose that the Na^+-independent anion antiporter (or a molecule closely linked to it) acts as the DT receptor and plays a role in its translocation to the cytosol. Furthermore, they suggest that an ion flux through the antiporter is associated with DT

TABLE III

Comparison of immunotoxin kinetics

Immunotoxin	Cell line	Sites/cell	K_a	Ref.	Conc	Lag time[a]	t_{10}[b]	First-order inactivation rate constant[c]	Immunotoxins bound/cell[d]	Rate constant/bound immunotoxin[e]
		n	M^{-1}		M	h	h	$K\ h^{-1}$	B	$(K \times 5 \times 10^5)/B$
UCHT1-DT	Jurkat	1×10^5	1×10^{10}	53, 16	5×10^{-10}	1.6	7.0	0.43	8.3×10^4	2.6
UCHT1-CRM 107	Jurkat	1×10^5	1×10^{10}	53, 16	3×10^{-10}	1.6	6.7	0.45	7.5×10^4	3.0
Tfn[f]-DT	K562	1.5×10^5	5.3×10^8	25	1×10^{-9}	1.2	2.1	2.5	5.2×10^4	24.2
Tfn-CRM 107	K562	1.5×10^5	5.3×10^8	25	1×10^{-9}	0.7	1.5	3.0	5.2×10^4	28.9
Tfn-DT	CEM	6×10^4	1×10^{12}	31	1×10^{-9}	1.8	4.8	0.8	6×10^4	6.3
Tfn-CRM 107	CEM	6×10^4	1×10^{12}	31	1×10^{-9}	1.1	3.3	1.1	6×10^4	9.0
Tfn-DT	Jurkat	6×10^4	1×10^{12}	[g]	1×10^{-10}	3.0	5.8	0.84	5.9×10^4	7.1[g]
Tfn-CRM 107	Jurkat	6×10^4	1×10^{12}	[g]	1×10^{-10}	1.8	4.0	1.05	5.9×10^4	8.9[g]
DT	Vero	1.6×10^5	1×10^9	3	1×10^{-9}	0.15	2.2	1.12	8×10^4	7.0
CRM 107	Vero	1.6×10^5	1.25×10^5	13	1×10^{-6}	0.8	4.2	0.67	8.9×10^4	3.8

[a] Lag time is the time required before first-order inactivation of protein synthesis begins.
[b] t_{10} is the time required for 90% inhibition of protein synthesis.
[c] The first-order rate constant for protein synthesis inhibition is: $\ln 10/(t_{10} - \text{lag time})$.
[d] Immunotoxins bound per cell were calculated by the following formula: $B = (nK_a(F))/(1 + K_a(F))$.
[e] The first-order rate constant is for 5×10^5 immunotoxins bound per cell.
[f] Tfn, transferrin.
[g] Values for n and K_a on Jurkat cells were assumed to be similar to those found on the other T-cell line, CEM (31). This may, however, represent an underestimation of the rate constant per bound immunotoxin. If the K_a on Jurkat is similar to that found on mixed peripheral mononuclear cells (4×10^7) (54), the rate constant per bound immunotoxin greatly increases.

translocation. Since we found that alternative cell-surface receptors are as effective or more effective in transporting the toxin to the cytosol than the native DT receptor, we examined the role of the anion antiporter in the translocation of the conjugates to the cytosol. Specifically, if toxins bind via other cell-surface receptors (T3, transferrin receptor, or Thy1) rather than via the anion antiporter, is it still necessary for the toxin to translocate through the anion antiporter to inactive protein synthesis?

CRM 107 conjugates enable us, for the first time, to investigate accurately the role of DT receptor binding in translocation. Binding of the conjugate is mediated by the new binding moiety and not by a combination of the new sites and the DT receptor (Table II). Therefore, the effect of SITS on CRM 107 conjugate toxicity will indicate the role of the anion antiporter in translocation, independent from its role in binding.

Various cell lines were preincubated with 1 mM SITS and then exposed to native DT, CRM 107, or the toxin conjugates. Sandvig and co-workers (4, 7) used Vero cells derived from monkey kidney to show that SITS blocked both binding and translocation of DT. Human transferrin, unlike many monoclonal antibodies, is able to cross species boundaries and to bind to monkey transferrin receptors (29). This therefore enables us to evaluate the effect of SITS on the transferrin-CRM 107 conjugate in an identical cell line as used for previously published reports. Fig. 2*A* shows that 1 mM SITS blocked DT toxicity 33-fold in a 5-h assay. In contrast, transferrin-CRM 107 exhibited only a 5-fold block of toxicity when cells were preincubated with SITS. The effect of SITS on the transferrin-toxin conjugates was also analyzed on Jurkat cells. Transferrin-CRM 107 was inhibited 10-fold by preincubating the cells with SITS, whereas native DT was inhibited 100-fold (Fig. 2*B*).

In a 16-h assay, again using Jurkat cells, SITS blocked the toxicity of UCHT1-CRM 107 only 2-fold and native DT 33-fold (Fig. 2*C*).

Jurkat cells, transfected with the mouse gene for Thy1.2 (15), were used to evaluate the effect of an immunotoxin generated by conjugating an anti-Thy1 antibody, 3A7, with DT (Fig. 2*D*). The 3A7-DT conjugate displayed a similar IC_{50} to that of native DT. However, preincubation with SITS produced very different effects on native DT than the immunotoxin. SITS blocked native DT 10-fold, but had no effect on the 3A7-DT immunotoxin.

We therefore found that, whereas DT was blocked 10–100-fold by SITS, three alternative receptors for DT (transferrin, T3, and Thy1) exhibited a 0–10-fold block on toxicity when preincubated with SITS. Thus, the potent toxicity of DT or CRM 107 linked to new binding moieties does not appear to depend on their translocation through the SITS-inhibitable anion antiporter.

Role of the Receptor in Murine Resistance to DT—The mouse T-cell lymphoma line EL-4 was assayed for its sensitivity to DT alone and the transferrin-CRM 107 conjugate. Characteristic of mouse cells, EL-4 cells are 1,000-fold less sensitive to DT toxicity than are K562 cells. Protein synthesis is inhibited 50% by 2.8×10^{-7} M DT (Fig. 3). This resistance is fully overcome by conjugating the toxin moiety to transferrin. The IC_{50} of transferrin-CRM 107 is 8×10^{-12} M, representing a 35,000-fold increase in toxicity over DT alone. Similar results were obtained using another murine cell line, WEHI-7; the IC_{50} of transferrin-CRM 107 was 4.8×10^{-12} M, 50,000-fold lower than that of native DT (data not shown).

The killing rate of transferrin-CRM 107 was compared for human and murine cell lines. Murine EL-4 cells have 3.9×10^4 transferrin receptors/cell (30), which is similar to the number of receptors found on human CEM cells (6×10^4) (31). If the affinity of human transferrin for EL-4 cells is as high as it is for CEM cells, then the rate constant per bound transferrin-CRM 107 conjugate is 6.3 for EL-4 cells (data not shown) compared to 9.0 for CEM cells (Table III). If the affinity of human transferrin for murine transferrin receptors

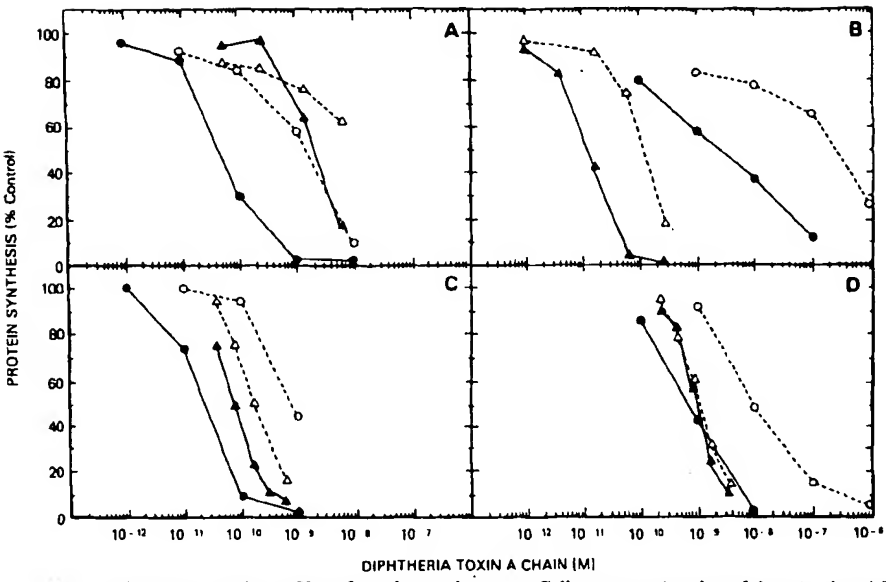

FIG. 2. **Effect of SITS on native DT and toxin conjugates.** Cells were preincubated for 30 min with 1 mM SITS. Control cells were preincubated with phosphate-buffered saline containing 0.2% bovine serum albumin. Preexposure of cells to SITS is indicated by *open symbols* and *broken lines*. Toxins were then added, and the cells were incubated for the specified amount of time, followed by a 1-h pulse with [^{14}C]leucine. *A*, Vero cells exposed to DT (●) or transferrin-CRM 107 (▲) for 24 h; *B*, Jurkat cells exposed to DT (●) or transferrin-CRM 107 (▲) for 5 h; *C*, Jurkat cells exposed to DT (●) or UCHT1-CRM 107 (▲) for 16 h; *D*, Jurkat cells transfected with Thy1 gene exposed to DT (●) or 3A7-DT (▲) for 5 h.

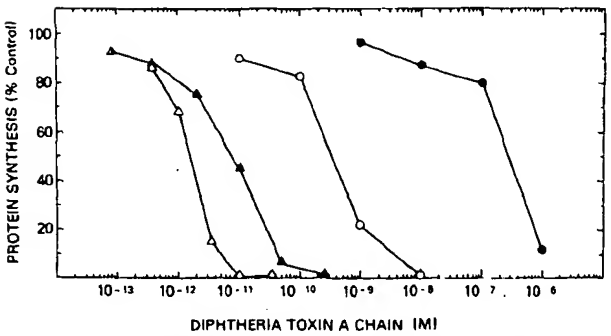

FIG. 3. **Response of EL-4 murine cells and K562 human cells to the transferrin-CRM 107 conjugate and native DT in a 24-h assay.** ○ and ●, DT; △ and ▲, transferrin-CRM 107; △ and ○, K562 cells; ▲ and ●, EL4 cells.

is lower than it is for humans, the rate constant for murine cells increases. In spite of this potential for underestimation, the kinetics of killing of transferrin-CRM 107 on murine cells is as efficient as that for DT on Vero cells. The fact that the natural resistance of murine cells to DT could be overcome by supplying a new binding moiety suggests that the defect in the mouse is at the level of the DT receptor and is not a defect in translocation activity.

DISCUSSION

CRM 107 has a phenylalanine at positions 390 and 525 of the DT sequence, resulting in a 10,000-fold reduction in toxicity and an 8,000-fold decrease in binding activity relative to native DT (13). In this report, we use the binding mutant CRM 107 to investigate the role of the receptor in the intoxication process. By linking several new binding moieties to CRM 107, we were able to investigate: 1) the role of the toxin-binding site in the entry of toxin conjugates; 2) the role of new DT receptors in the kinetics of entry to the cytosol; 3) the role of the anion antiporter, the proposed DT receptor, in the translocation of toxin conjugates; and 4) the nature of the defect in mouse that results in resistance of mouse cells to DT.

The kinetics of transferrin and UCHT1 linked to DT and CRM 107 were compared with native DT. Binding moieties linked to DT or CRM 107 had comparable killing rates, indicating that altering the binding site of the toxin portion of the conjugate had no effect on the kinetics of intoxication. However, the binding moiety (DT, transferrin, or UCHT1) was found to determine the ratio of the lag period to the killing rate.

The transferrin-toxin conjugates are characterized by an extremely long lag period, followed by a rapid killing rate. The dose-dependent lag time before the inhibition of protein synthesis begins is thought to represent a processing event (26, 32, 33). Transferrin has extremely rapid internalization and recycling, with the entire transferrin cycle taking only 4–5 min in K562 cells (34, 35). Up to 30 successive cycles of transferrin-CRM 107 recycling may be possible before protein synthesis inhibition occurs. Alternatively, the conjugate may be internalized by another transferrin route (36–38) that requires a longer time to initiate intoxication.

The anion antiporter has been proposed to be the site for both DT binding and translocation (4–8). However, we found that alternative binding moieties were as effective as the native DT receptor in the translocation of the toxin to the cytosol. We therefore investigated the role of the anion antiporter in the binding and translocation of the conjugates. We observed a 10–100-fold block of native DT by SITS. In contrast, we found that three alternative receptors for DT (transferrin, T3, and Thy1) were not blocked or were blocked to a lesser degree by SITS (0–10-fold). We conclude that the potent toxicity of DT or CRM 107, when linked to new binding moieties, does not depend on their translocation through the SITS-inhibitable anion antiporter. SITS has been

reported to produce an increase in intracellular pH in other cell systems (39, 40). Therefore, the small blocking of DT conjugates observed with SITS may result from alterations in intracellular vesicle pH or other pleiotropic effects of SITS on cell physiology.

We used transferrin-CRM 107 to investigate the nature of the resistance to DT found in mouse cells. There are conflicting reports in the literature attributing the nature of the resistance to a defect at the receptor binding level (41, 42), at the receptor affinity level (43), or at the translocation level (44). The mouse resistance to DT was fully overcome by conjugating CRM 107 with transferrin, thereby allowing toxin binding to be mediated by the transferrin. Similar results were observed by transferrin-DT conjugates (45). These results demonstrate that the DT-binding sites, shown to exist on murine cells (44, 46, 47), are not functional receptors in that they do not lead to intoxication in cells fully capable of translocating DT to the cytosol via alternative receptors.

Our results are consistent with a simple model of DT entry into cells. First, diphtheria toxin binds cell surface receptors via the C-terminal region of the B subunit (48) involving amino acids 508 and either 525 or 390 (13). A second essential step is endocytosis to low pH compartments. This was first indicated by the fact that NH_4Cl and other reagents that increase the pH of intracellular compartments block DT toxicity (49) and more recently by the demonstration that exposure of cells to low pH circumvents this block (50, 51). The extremely efficient killing rate of transferrin-CRM 107 may be reflective of the close parallels between DT and transferrin endocytosis and processing. A low pH environment is required for the diferric form of transferrin to release its iron and for apotransferrin to be recycled back to the cell surface. By linking CRM 107 with transferrin, we have not only provided a new binding moiety but have also selected a receptor-ligand complex that is rapidly internalized to an environment that facilitates toxin translocation. Translocation out of the endosome to the cytosol appears to be carried out by the DT B chain because DT conjugates that lack the B subunit, such as UCHT1-DTA chain, are 10,000-fold less toxic than those that contain the B chain (52). The translocation step is enhanced 100-fold by the N-terminal half of the DT B chain and a further 100-fold in conjugates that also contain the C-terminal half of the B chain (52). This step does not appear to require the DT B chain-binding site because CRM 107 conjugates are equal in translocation activity to conjugates with native B chain.

Antibody and transferrin-CRM 107 conjugates are unique and powerful reagents to examine DT toxicity because each of four steps is carried out by a separate domain. Cell-surface binding is mediated by the antibody or transferrin portion of the toxin conjugate, whereas endocytosis to low pH compartments is mediated by the cell-surface receptor. Translocation across the intracellular compartment membrane to the cytosol is performed by the B subunit of CRM 107, and inactivation of protein synthesis is mediated by the A subunit. The toxin conjugates allow the pathway to be dissected and the role of the individual functions to be separated and studied independently and quantitatively.

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Improved tumor-specific immunotoxins in the treatment of CNS and leptomeningeal neoplasia

VIRGINIA G. JOHNSON, PH.D., CHARLES WROBEL, M.D., DEBRA WILSON, B.S., JOHN ZOVICKIAN, M.D., LARRY GREENFIELD, M.D., EDWARD H. OLDFIELD, M.D., AND RICHARD YOULE, PH.D.

Surgical Neurology Branch, National Institute of Neurological Communicative Diseases and Stroke, National Institutes of Health, Bethesda, Maryland; and Department of Microbial Genetics, Cetus Corporation, Emeryville, California

✓ A novel antibody-toxin conjugate has been developed for use in cancer therapy. This report demonstrates that this new reagent selectively kills glioblastoma- and medulloblastoma-derived cell lines, medulloblastoma cells in primary culture, and cell lines derived from tumors commonly metastatic to the cerebrospinal fluid (CSF). Efficient killing of human tumor cells occurred at concentrations between 3.9×10^{-13} M and 1.1×10^{-10} M, whereas guinea pigs and rhesus monkeys tolerated intrathecal levels of 2×10^{-9} M. Cerebrospinal fluid from normal humans and from brain-tumor patients does not inhibit the *in vitro* efficacy of this reagent. The wide therapeutic window, extreme potency, and general applicability of this antibody-toxin conjugate against CSF-borne primary or metastatic tumors warrants clinical trials.

KEY WORDS • **diphtheria toxin** • **immunotoxin** • **transferrin receptor** • **leptomeningeal neoplasia** • **brain neoplasm** • **cerebrospinal fluid**

CURRENT treatments utilizing surgery, radiation therapy, and systemic chemotherapy have done little to alter the natural outcome of many malignant tumors of the central nervous system (CNS). Immunotoxins (protein toxins chemically linked to tumor-specific monoclonal antibodies or other ligands) offer potential advantage over more conventional forms of treatment by having higher tumor specificity.

Immunotoxins may be particularly efficacious for the treatment of neoplastic disease confined to compartments such as the peritoneum or intrathecal space. Direct delivery into the compartment avoids complications associated with systemic delivery and produces relatively high local concentrations, thereby achieving greater therapeutic effects. The cerebrospinal fluid (CSF) compartment may be amenable to this type of compartmentalized immunotoxin treatment. Zovickian and Youle[34] examined the therapeutic effect of a monoclonal antibody-ricin immunotoxin, delivered directly into the CSF compartment in a guinea pig model of leptomeningeal neoplasia. Immunotoxin therapy extended survival, corresponding to a 2 to 5 log kill of tumor cells, without detectable toxicity.

Protein toxins used in the construction of immunotoxins have an A and B subunit. The A subunit catalyzes the inactivation of protein synthesis, resulting ultimately in cell death. The B subunit has two functions: it is responsible for toxin-binding to the cell surface, and it facilitates the translocation of the A chain across the membrane and into the cytosol, where the A chain acts to kill cells (Fig. 1). Previously, two general types of immunotoxins have been used. Immunotoxins made with the complete toxin molecule (A and B chains) have the complication of nonspecific killing mediated by the toxin B chain binding site. This can be avoided by eliminating the B chain and linking only the A chain to the antibody. However, A chain immunotoxins, although more specific, are much less toxic to tumor cells.[4] In addition to a binding function, the B chain has an entry function, which facilitates the translocation of the A chain across the membrane and into the cytosol (Fig. 1). Since A chain immunotoxins lack the entry function of the B chain, they are less toxic than their intact toxin couterparts containing the complete B chain. An ideal toxin for immunotoxin construction would contain the A chain enzymatic function and the B chain translocation function, but not the B chain binding function.

We have recently described a new, genetically engineered toxin that offers these major advantages.[9,12] This

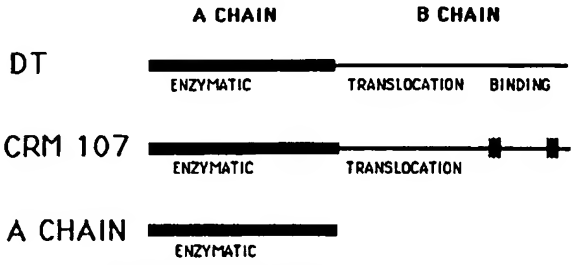

FIG. 1. Diagrammatic representation of diphtheria toxin (DT), CRM 107, and DT A chain structure and function. Native DT is composed of an A and B subunit, with the A chain containing the enzymatic function and the B chain containing the binding and translocation functions. Two point mutations in CRM 107 inactivate the toxin binding function but leave the translocation and enzymatic functions intact. Toxin A chain contains only the enzymatic function.

toxin, called CRM 107, is identical to diphtheria toxin (DT) except for two amino acid changes in the B chain. These two point mutations inactivate toxin binding 8000-fold but leave the translocation function intact. Immunotoxins made with CRM 107 therefore have the advantage of the lower nontarget-cell toxicity of A chain conjugates but retain a 10,000-fold increase in tumor-specific toxicity similar to that found for immunotoxins made with complete toxin molecules.[9]

The tremendous potential of CRM 107 for use in immunotoxins together with the advantage of CSF compartmentalized delivery may result in effective treatment for tumors in the CNS. The transferrin receptor (TfR) has been chosen as the target for these immunotoxins. Expression of TfR increases during cell replication in order to fulfill the dividing cell's need for iron. Since tumor cells are dividing while normal cells in the CNS parenchyma are quiescent, the TfR should provide an effective target for immunotoxin therapy. It has previously been shown that TfR's are expressed on human medulloblastoma and glioblastoma cells.[33] This report demonstrates that transferrin (Tf) or an anti-TfR monoclonal antibody conjugated to CRM 107 has potential for effective treatment of medulloblastoma and breast leptomeningeal carcinomatosis.

Materials and Methods

Established Cell Lines

The SNB75 cell line was established by primary explants from a tumor removed from a 72-year-old woman with a bifrontal glioblastoma multiforme. SNB101 was also established by primary explants from a glioblastoma multiforme removed from the right parietal lobe of a 49-year-old man. SNB40 was derived from primary explants of a medulloblastoma surgically removed from the posterior fossa of an 8-year-old boy. At the time of this study, SNB75 was in its 48th passage, SNB101 in its 14th passage, and SNB40 in its 20th passage. U251 is a cell line of human glioma origin adapted to culture by J. Pontén and B. Westermark at the University of Uppsala, Uppsala, Sweden.[20,29] At the time of this study, U251 was in its 37th passage. TE671* is derived from a human medulloblastoma.[18] All of these cell lines were maintained in Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10 μg/ml gentamicin.

Three cell lines from human breast cancers were examined. MCF-7, a breast adenocarcinoma-derived cell line,[24] was maintained in DMEM containing the supplements described above plus 10 μg/ml insulin. ZR-75-1, a cell line derived from a malignant ascitic effusion of a patient with infiltrating ductal carcinoma,[5] was maintained in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 20 μg/ml gentamicin, and 2 mM glutamine. T47D, a cell line derived from an infiltrating ductal carcinoma,[13] was grown in RPMI 1640 medium as described above with the addition of 10 μg/ml insulin. All breast-derived cell lines were a gift from Dr. J. Greiner and Dr. J. Schlom.

Primary Medulloblastoma Cultures

Since established cell lines long adapted to culture conditions could conceivably possess Tf requirements and receptor levels different from those of the original cells, we established two primary medulloblastoma cultures and determined sensitivity to Tf-CRM 107. The SNB104 cell line was established from biopsy of a midline cerebellar vermian mass in an 18-year-old man, and SNB105 was derived from a midline posterior fossa tumor in a 5-year-old girl. In both cases, fresh tumor was transported to the laboratory in Eagle's minimum essential medium (MEM) with 10% FCS. Tumors were mechanically and then enzymatically dissociated according to previously described techniques[11] and cultured at a density of 1 to 5 $\times$ 10^5 cells/ml in 75 sq cm tissue culture flasks. The culture medium consisted of Eagle's MEM with Earle's salts supplemented with 10% FCS, 1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin (100,000 μ/liter), streptomycin (100 mg/liter), and Fungizone (amphotericin B, 0.25 mg/liter). Cultures attained 50% confluence within 10 to 14 days, at which time trypsinization, transfer to 96 well plates, and cytotoxicity testing were performed as described below.

Synthesis and Purification of CRM 107 Immunotoxins

CRM 107, isolated by Laird and Groman,[14] was purified as described previously.[12] Human Tf† was loaded with iron according to the method of Shindelman, *et al.*[23] Conjugation of Tf with CRM 107 was accomplished by first generating free sulfhydryl groups on Tf with 2-iminothiolane, which was dissolved in 0.8 boric acid (pH 8.5) and incubated with Tf in an 8:1

* TE671 cell line obtained from American Type Culture Collection, Rockville, Maryland.

† Human transferrin obtained from Sigma Chemical Co., St. Louis, Missouri.

molar ratio. The modified Tf was kept at room temperature for 1 hour, then separated from free 2-iminothiolane by gel filtration on a Sephadex G-25 gel filtration column equilibrated with phosphate-buffered saline (PBS). The bifunctional cross-linking agent, *M*-maleimido-benzoyl-N-hydroxysuccinimidyl (MBS) ester, was used to link Tf to CRM 107; MBS was dissolved in dimethylformamide and added in five-fold molar excess to the toxin. The mixture was incubated for 30 minutes at room temperature, then separated by chromatography on a Sephadex G-25 gel filtration column. The MBS-conjugated toxin was mixed with thiolated Tf in 1:1.3 molar ratio and incubated for 3 hours at room temperature; the toxin-conjugate was then purified by gel filtration on a TSK-3000 high-performance liquid chromatography column. One-minute fractions were collected and individual fractions were tested for toxicity using protein synthesis inhibition. Peak fractions of the toxin conjugate were pooled and then A chain activity in this pooled peak was quantified using the elongation factor 2 (EF-2) adenosine diphosphate (ADP)-ribosylation assay. This pooled peak was used for all further experiments.

The anti-TfR monoclonal antibody, 454A12, was prepared as previously reported.[8] It was linked to CRM 107 as described above. This antibody was also conjugated with recombinant ricin toxin A chain (RTA) as described by Bjorn, *et al.*[2]

ADP-Ribosylation Assay

The concentration of the CRM 107 immunotoxins was determined using an ADP-ribosylation assay. This assay measures the ability of the CRM 107 immunotoxins to catalyze the transfer of ADP-ribose from nicotinamide-adenine dinucleotide (NAD) to EF-2.

Elongation factor 2 was purified from rat liver cells following a procedure described previously.[3] ADP-ribosylation was carried out in 80-μl reaction mixtures containing 40 μl 0.01 M Tris-HCl buffer, with 1.0 M dithiothreitol (pH 8.0), 20 μl EF-2, and 10 μl toxin sample. The reaction was initiated by the addition of 10 μl of phosphate-32-labeled NAD (1.2 μCi, specific activity 277 Ci/mmol, adjusted to 180 μM with cold NAD). Reaction mixtures were incubated at room temperature for 20 minutes and the reaction was stopped by the addition of 1 ml 10% trichloroacetic acid (TCA). The precipitate was washed once with 10% TCA, solubilized in 0.1 M NaOH, and counted.

The ADP-ribosylation activities of unknown samples were compared to values obtained from DT standards consisting of known concentrations of DT (values based on Lowry's protein determination[16] using bovine serum albumin (BSA) as a standard). The background of the assay was determined by replacing the toxin with Tris-HCl buffer.

Protein Synthesis Assay

Inhibition of protein synthesis was used to assay the cytotoxic effects of the toxin-conjugate. Cells were trypsinized, washed in their regular growth medium, and dispersed in this medium into 96-well microtiter plates at a density of 5×10^4 cells per well. The cells were allowed to reattach and grow in the 96-well plate for 24 hours before the assay was performed. They were then washed twice with leucine-free RPMI medium containing 10 mM HEPES and 10 μg/ml gentamicin but without FCS, and the wells were refilled to a final volume of 100 μl. Toxin-conjugates, toxins alone, or control solutions were added to the wells in 11-μl aliquots and the cells were incubated at 37°C for 24 hours. At the end of this time, 20 μl of PBS containing 0.1 μCi of carbon-14 (^{14}C)-labeled leucine was added, incubation continued for 1 hour, and the cells were harvested onto glass fiber filters using a PHD cell harvester.‡ The filters were washed with water, dried, and counted. All cytotoxicity assays were performed two to five times in triplicate. The results were expressed as a percentage of ^{14}C-leucine incorporation in mock-treated control cultures.

In Vivo *Toxicity*

Guinea Pig. To investigate the efficacy of intrathecal immunotoxin therapy for tumors of the CSF compartment, we determined the toxicity of DT or CRM 107 alone, or Tf-CRM 107 conjugate injected directly into the cisterna magna of Strain 2 guinea pigs. The animals were anesthetized with intraperitoneal ketamine (30 to 50 mg/kg). Toxin or conjugate, suspended in 100 μl PBS/0.2% BSA, was slowly injected percutaneously via a No. 25 needle into the cisterna magna. Injections were performed only after CSF was clearly visualized in the hub of the needle. Final concentrations achieved in the CSF were calculated based on a total guinea pig CSF volume of 500 μl. The length of survival was recorded as the number of days following injection until death. Body weight was also measured at designated intervals and compared to that of control animals injected with PBS alone.

Rhesus Monkey. A single dose of Tf-CRM 107 in 0.5 cc normal saline containing 0.2% human serum albumin was administered to two anesthetized adult rhesus monkeys by an occiput-C-1 puncture and gentle barbotage. This technique is known to effect rapid mixing of injectant with cisternal and ventricular CSF.[7] Venous blood for chemistry and hematology panels and lumbar CSF for routine studies were obtained every other day for 1 week and weekly thereafter. The animals were weighed at the same time that blood and CSF were collected.

Results

Toxicity of CRM 107 Immunotoxins on Human Tumor-Derived Cell Lines

Diphtheria toxin or DT-like toxins such as CRM 107 inhibit protein synthesis. This process can be measured

‡ Cell harvester manufactured by Cambridge Technology, Inc., Cambridge, Massachusetts.

for an accurate *in vitro* assessment of the lethal effect of the toxin on cells.

Figure 2A shows representative dose-response curves of the Tf-CRM 107 conjugate on four medulloblastoma-derived cell lines. A steep dose-response inhibition of protein synthesis by Tf-CRM 107 was observed with all the cell lines. Protein synthesis in SNB40 cells was blocked by Tf-CRM 107 at a 50% inhibiting concentration (IC_{50}) of 3.9×10^{-13} M (Table 1). TE671 cells and the primary medulloblastoma-derived cell line, SNB104, were also extremely sensitive to Tf-CRM 107 (IC_{50}:2.5×10^{-12} M and 2.1×10^{-12} M, respectively). The IC_{50} of Tf-CRM 107 for the other primary medulloblastoma derived cell line, SNB105, was 1.1×10^{-10} M. The receptor specificity of the Tf-CRM 107 conjugate was demonstrated by the fact that excess free Tf blocked cell killing by the toxin conjugate (data not shown).

Figure 2B shows the results of similar experiments performed on the glioblastoma-derived cell lines. As observed with cells derived from medulloblastoma, Tf-CRM 107 exhibited potent killing with all the glioblastoma cells. The IC_{50} for SNB75 was 6.5×10^{-11} M, and the IC_{50} for SNB101 and U251 was 5.4×10^{-12} M and 2.6×10^{-12} M, respectively. Using a monoclonal antibody against the human TfR, 454A12, linked to CRM 107 in trials with two continuous medulloblastoma cell lines and three glioblastoma cell lines showed IC_{50} levels between 10^{-11} and 10^{-10} M, whereas, when this same conjugate was assayed on Vero cells, which lack the receptor, the IC_{50} level was 1×10^{-8} M.[9] The therapeutic window between tumor and nontarget cells is therefore 100- to 1000-fold. CRM 107 and RTA were assayed as free toxins, as shown in Table 1. Both free toxins displayed similar toxicities and were 1000 to 10,000-fold less toxic than the 454A12-CRM 107 immunotoxin. The wide therapeutic windows between target and nontarget cells and between free toxin and immunotoxin demonstrate that the 454A12-CRM 107 is highly potent and specifically toxic to brain-tumor cells.

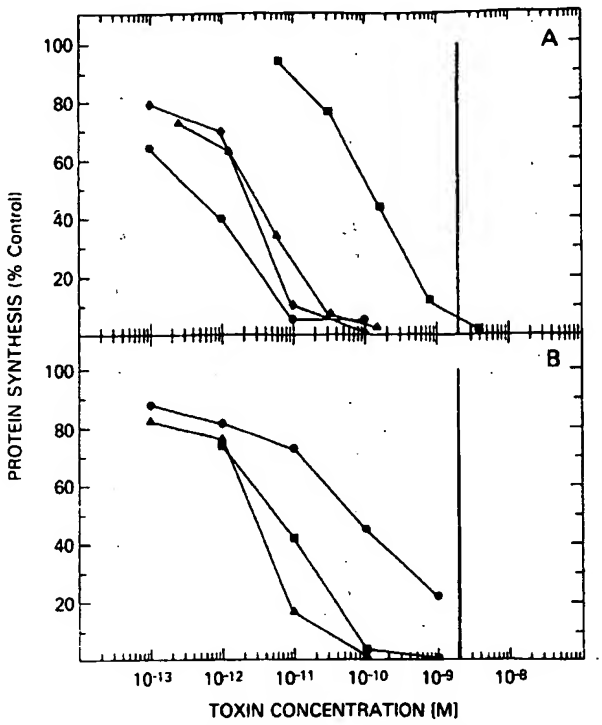

FIG. 2. Cytotoxic effects of transferrin (Tf)-CRM 107 on cells derived from medulloblastoma and glioblastoma compared to maximum tolerable levels achieved in the cerebrospinal fluid of rhesus monkeys. Primary cultures or established cell lines were incubated for 24 hours with varying concentrations of Tf-CRM 107 followed by 1 hour of incubation with 0.1 μCi ^{14}C-leucine. Cells from triplicate cultures were then harvested and protein synthesis in treated cells was expressed as a percentage of ^{14}C-leucine incorporated into untreated control cells. Concentrations of 2×10^{-9} M of Tf-CRM 107, injected intrathecally into the cisterna magna of rhesus monkeys, could be reached safely *(vertical line)*. A: Dose-response curves for medulloblastoma-derived cells. *Squares:* SNB105; *diamonds:* SNB104; *triangles:* TE671; *circles:* SNB40. B: Dose-response curves for glioblastoma-derived cells. *Circles:* SNB75; *triangles:* U251; *squares:* SNB101.

TABLE 1

*IC_{50} values for CRM 107 and ricin A chain (RTA) immunotoxins**

Tumor Cells	Tf-CRM 107	454A12-CRM 107	454A12-RTA	CRM 107	RTA
medulloblastoma					
SNB40 (established)	3.9×10^{-13}	3.5×10^{-11}	3.3×10^{-11}	3.0×10^{-7}	1.1×10^{-7}
TE671 (established)	2.1×10^{-12}	2.6×10^{-11}	2.6×10^{-11}	2.8×10^{-7}	2.0×10^{-7}
SNB104 (primary)	2.5×10^{-12}	not done	not done	not done	not done
SNB105 (primary)	1.1×10^{-10}	not done	not done	not done	not done
glioblastoma					
SNB75	6.5×10^{-11}	1.2×10^{-10}	1.5×10^{-10}	1.6×10^{-7}	5.5×10^{-7}
SNB101	5.4×10^{-12}	3.8×10^{-11}	3.0×10^{-10}	4.0×10^{-8}	5.0×10^{-7}
U251	2.6×10^{-12}	1.6×10^{-11}	3.6×10^{-11}	2.1×10^{-7}	8.2×10^{-8}
breast carcinoma					
MCF-7	2.3×10^{-11}	1.2×10^{-10}	3.2×10^{-10}	6.0×10^{-7}	1.1×10^{-7}
T47D	1.1×10^{-12}	1.0×10^{-10}	7.0×10^{-11}	1.3×10^{-6}	2.2×10^{-7}
ZR-75-1	1.6×10^{-11}	2.1×10^{-10}	3.8×10^{-10}	2.8×10^{-7}	1.8×10^{-7}

* Immunotoxins and toxins were incubated with the cells for 24 hours followed by incubation of 1 hour with ^{14}C-leucine. Cells were then harvested and concentrations of immunotoxin that inhibit protein synthesis by 50% of control values (IC_{50}) were determined. Tf = transferrin.

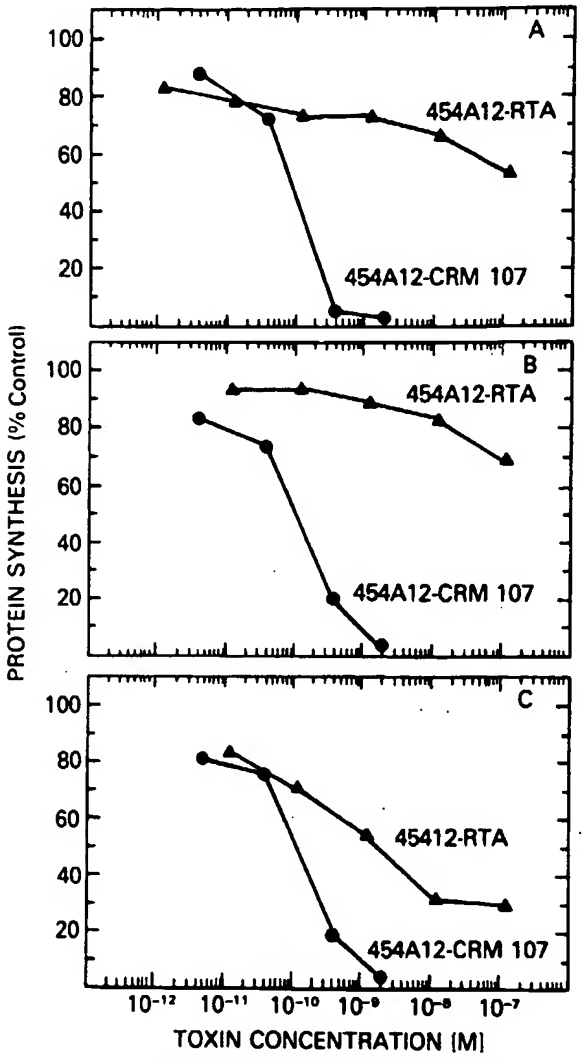

FIG. 3. Comparison of the toxicity of 454A12 monoclonal antibody linked to CRM 107 or to ricin A chain. Cell lines derived from medulloblastoma (TE671 cell line, A), glioblastoma (U251 cell line, B), or breast carcinoma (T47D cell line, C) were incubated with 454A12 immunotoxins for 3 hours followed by a 1-hour pulse with ^{14}C-leucine. Cells were harvested and protein synthesis in treated cells was expressed as a percentage of ^{14}C-leucine incorporated into untreated control cells.

Toxicity of Anti-TfR Antibody Conjugated to Ricin A Chain and to CRM 107

The toxicity of the anti-TfR antibody, 454A12, conjugated to recombinant ricin A chain (RTA) or to CRM 107 was compared with Tf-CRM 107 on cell lines derived from medulloblastoma and glioblastoma (Table 1). Both the 454A12-RTA and the 454A12-CRM 107 immunotoxin displayed very similar toxicities when evaluated after 24 hours using the *in vitro* assay of protein synthesis described above. The IC_{50} observed for both of these immunotoxins on established medulloblastoma and glioblastoma cell lines ranged from approximately 10^{-10} to 10^{-11} M. Only one cell line, SNB101, displayed a significant difference in sensitivity to the two immunotoxins. For this cell line, 454A12-CRM 107 was approximately 10-fold more toxic than 454A12-RTA.

Three established breast-derived cell lines, MCF-7, T47D, and ZR-75-1, were assayed, since breast tumors most commonly lead to meningeal carcinomatosis (Table 1). These cells displayed approximately the same sensitivity to the immunotoxins as was found for the medulloblastoma or glioblastoma cells. In a 24-hour assay, the IC_{50} was similar for both 454A12-RTA and 454A12-CRM 107, ranging between 1×10^{-10} M and 7×10^{-11} M. Tf-CRM 107 was approximately 10- to 100-fold more toxic than the 454A12 immunotoxins on these breast cell lines.

Figure 3 demonstrates very different dose-response curves for 454A12-CRM 107 and 454A12-RTA after 3 hours of incubation with the immunotoxins on representative cell lines derived from medulloblastoma, glioblastoma, and breast carcinoma. The 454A12-CRM 107 displayed a steep dose-response curve for all three cell lines, with an IC_{50} between 1×10^{-10} M and 6×10^{-10} M. The RTA immunotoxin is less toxic, inhibiting protein synthesis by less than 30% in TE671 and U251 cells at concentrations greater than 10^{-7} M. 454A12-RTA is approximately 15-fold less toxic to T47D breast carcinoma cells than the CRM 107 immunotoxin.

Similar dose-response curves for 454A12-CRM 107 and 454A12-RTA were obtained in a 24-hour assay (Table 1), yet very different curves result from a 3-hour assay with the two toxin conjugates (Fig. 3). This indicates that large differences exist in the rate of cell killing by the two immunotoxins. Kinetic differences in the rate of killing by immunotoxins can be masked in an assay where inhibition of protein synthesis is measured over a long incubation period. The assay cannot detect cell killing beyond 90% of the input cells (10% of control protein synthesis). Immunotoxins with rapid killing may reach this level quickly and continue to kill additional logs of cells that are not detected in the assay. After long incubation times, the dose-response curves of immunotoxins with efficient killing rates may appear identical to those with less efficient rates of kill, since the assay does not reflect the additional log kill.

Maximum Tolerable Dose In Vivo

Guinea Pigs. To investigate the feasibility of intrathecal immunotoxin therapy for tumors in the CSF, the toxicity of DT, CRM 107, and Tf-CRM 107 was determined. Varying concentrations of the toxins were injected percutaneously into the cisterna magna of guinea pigs. The maximum safe dose (maximum dose where no significant weight loss was observed) of DT was between 3.2×10^{-11} M and 3.2×10^{-12} M (Table 2). Up to 100-fold higher doses of CRM 107 were tolerated without detectable toxicity. Therefore, the toxicity of CRM 107 *in vivo* is 1/100 that of DT, whereas the nonspecific toxicity of CRM 107 *in vitro*

TABLE 2

*Maximum tolerated dose in guinea pig cerebrospinal fluid**

Toxin	Concentration
diphtheria toxin	3.2×10^{-11} M to 3.2×10^{-12} M
CRM 107	3.2×10^{-10} M
Tf-CRM 107	2×10^{-9} M

* Toxin alone or transferrin (Tf)-CRM 107 conjugate was injected percutaneously into the cisterna magna of Strain 2 guinea pigs. The maximum dose consistent with survival is shown. No significant weight loss was observed in animals treated at these doses when compared with control animals.

is 1/10,000 that of DT.[9] Furthermore, conjugation of CRM 107 to Tf reduced the toxicity approximately 10-fold more (2×10^{-9} M). Inactivation of toxicity due to conjugation was previously observed with other immunotoxins.[32]

Rhesus Monkeys. Tf-CRM 107 was injected intrathecally into the cisterna magna of two adult rhesus monkeys. Assuming a volume of distribution of 6 cc,[17] doses were administered to produce a CSF concentration of 3.3×10^{-10} M and 2×10^{-9} M. Neither dose of Tf-CRM 107 caused apparent neurological illness, and both animals were alive 2 months after treatment. Weight loss was limited to less than 10% of baseline. At both dose levels, prominent fevers (> 39.5°C) occurred on Days 1 and 2 following treatment. Serum chemical levels, liver enzyme concentrations, renal function, and hematological parameters did not change. A CSF inflammatory response with pleocytosis, elevated protein levels, and normal glucose content was apparent for 48 hours but had largely resolved by 14 days after treatment.

The concentration of 2×10^{-9} M, which was reached safely *in vivo*, was 20 to 5000 times greater than the IC_{50} of Tf-CRM 107 to all the medulloblastoma, glioblastoma, and breast cells assayed in culture.

Inhibiting Effects of Circulating Anti-DT Antibodies

A critical factor in the efficacy of any CRM 107 immunotoxin in man is the level of inactivating anti-DT immunoglobulin produced by intentional immunization with diphtheria toxoid. Since CRM 107 differs from DT in only two amino acids, it is expected that the majority of circulating antibodies would be cross-reactive with CRM 107. We therefore investigated the effect on DT toxicity of circulating levels of antibody in the serum and CSF.

As shown in Fig. 4A, most human sera contain significant titers of inactivating antibody. Further titration of the sera with higher levels of DT revealed approximately a 10,000-fold block by sera (data not shown). Serum samples from two donors, reportedly not intentionally immunized against DT, exhibited dose-response curves that closely paralleled the control DT curve; these samples serve as controls showing that human sera have no other effects on DT toxicity.

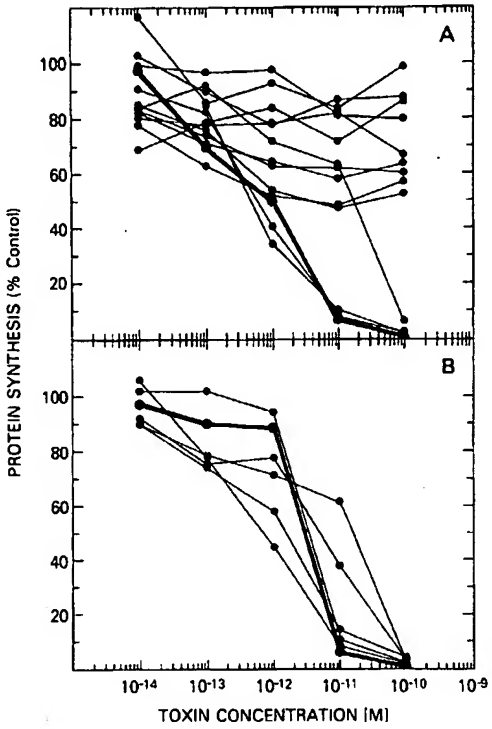

FIG. 4. Inhibition of diphtheria toxin (DT) cytotoxicity by human serum or cerebrospinal fluid (CSF). Vero cells, derived from green-monkey kidney, have high levels of DT receptors (1.6×10^5 sites/cell)[19] and as a result are extremely sensitive to DT toxicity. Vero cells were therefore used to determine the presence of anti-DT antibodies in the serum and CSF of normal volunteers. Dilutions of DT were preincubated at room temperature with an equal volume of undiluted serum or CSF for 30 minutes, then 22 μl of the preincubated DT mixture was added to the Vero cells and incubated for 14 hours. Cytotoxicity assays were performed and results expressed as described in Fig. 1 and in the Materials and Methods section. Results with a DT control sample (preincubated with phosphate-buffered saline rather than serum or CSF) are plotted as a *bold line*. The effect of preincubation of DT with serum (A) or CSF (B) from normal volunteers is shown by the *thin lines*.

Low levels or total absence of inactivating antibody was found in the CSF (Fig. 4B) of normal volunteers. The CSF from a glioblastoma patient, a patient with breast cancer-related leptomeningeal carcinomatosis, and a patient with lymphomatous leptomeningitis was also tested (data not shown) and showed no inhibition of DT toxicity. The fact that CSF has only 0.2% to 0.4% of the immunoglobulin G levels found in serum[22] is consistent with our results which also substantiate the belief that the CSF compartment is an immunologically privileged site.

Inhibition of TF-CRM 107 and 454A12-CRM 107 by Free Tf

It is reported that CSF has 14 μg/ml of circulating Tf.[6] Therefore, the effect of this concentration of free

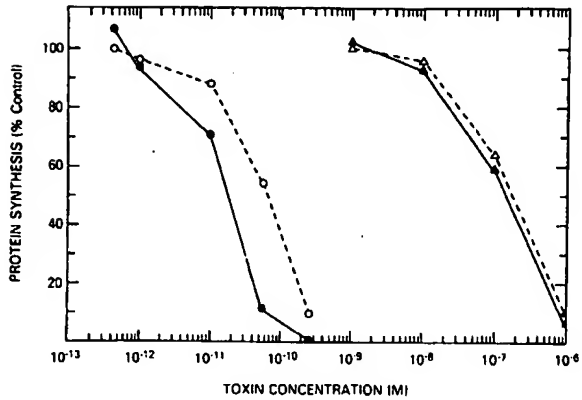

FIG. 5. Inhibition of cytotoxicity by free transferrin (Tf). The human erythroblastoma-derived cell line, K562, has been shown to express high levels of Tf receptor (1.5×10^5 sites/cell)[20] and as a result is extremely sensitive to the Tf-CRM 107 conjugate. The effect of Tf at 14 μg/ml, reported to be the level found in human cerebrospinal fluid, on the cytotoxicity of diphtheria toxin (DT) and Tf-CRM 107 was therefore evaluated using K562 cells. Cells in leucine-free media without fetal calf serum were incubated with diferric Tf, producing a final concentration of 14 μg/ml. Phosphate-buffered saline was added to the control wells. Dilutions of Tf-CRM 107 or native DT were added to the cell solution and the mixture was incubated for 5 hours at 37°C. Cytotoxicity assays were performed and results expressed as described in Fig. 1 and the Materials and Methods section. *Closed circles:* Tf-CRM 107; *open circles:* Tf-CRM 107 + 14 μg/ml Tf; *closed triangles:* DT; *open triangles:* DT + 14 μg/ml Tf.

Tf on the toxicity produced by Tf-CRM 107 was tested. The human erythroblastoma-derived cell line, K562, has been shown to express high levels of TfR (1.5×10^5 sites/cell),[25] and as a result is extremely sensitive to the Tf-CRM 107 conjugate. Therefore, using K562 cells, free Tf (14 μg/ml) was found to inhibit the Tf-CRM 107 conjugate fourfold and to have no effect on native DT (Fig. 5). Therefore, circulating levels of Tf in the CSF are sufficient to cause only a very minor block of toxicity.

Binding of 454A12-RTA and 454A12-CRM 107 was mediated by the antibody portion of the conjugate as shown by the fact that free antibody could block toxicity (data not shown). Binding of 454A12 to the TfR is not inhibited by free Tf; Tf found in the CSF at 14-μg/ml concentrations had no effect on the toxicity of either 454A12 immunotoxin.

Discussion

In this report, the potential of a new type of toxin, CRM 107, has been demonstrated for use in the construction of highly potent immunotoxins directed specifically at tumors of the CNS. Immunotoxins may be most effective for the regional treatment of disease confined to an anatomic compartment where transvascular delivery is not a problem and where relatively high local concentrations, and therefore greater therapeutic effect, may be achieved. It was previously shown that an anti-idiotype monoclonal antibody-ricin immunotoxin, delivered intrathecally, significantly extended survival in a guinea pig model of leptomeningeal neoplasia. The increased survival time, which corresponded to a median 2- to 3-log kill of tumor cells, occurred without detectable toxicity related to the immunotoxin.[34]

CRM 107 represents a significant advance in the design of toxins for use in immunotoxin therapy. This is a genetically modified form of DT, differing from native DT at amino acid positions 390 and 525.[9] The toxin molecule consists of an A and B subunit. The A subunit enzymatically inactivates protein synthesis by transferring ADP-ribose to EF-2, thereby stopping the addition of amino acids to the growing polypeptide chain and thus killing the cell. The B subunit has two functions, facilitating both the binding of the toxin to the cell surface and the entry or translocation of the A subunit across the cell membrane into the cytosol where it functions. The advantage of CRM 107 is that the two amino acid changes in the toxin B chain inactivate toxin binding 8000-fold yet have no effect on the translocation function.[9] Therefore, by linking CRM 107 to a specific binding moiety such as a tumor-specific monoclonal antibody, it is possible to target the full toxicity of the native toxin and still avoid the problems of nonspecific toxicity caused by toxin binding.

The advantages of CRM 107-based immunotoxins become apparent when compared with immunotoxins made with DT A chains alone. Colombatti, *et al.*,[4] compared the toxicity of native DT conjugated with a monoclonal antibody specific for the T lymphocyte antigen receptor with that of the DT A chain conjugated to the same antibody. It was found that A chain immunotoxins were 10,000-fold less toxic than those made with native DT. This reduction in toxicity reflects the loss of the B chain translocation function. CRM 107 retains the translocation function, and therefore, when linked to a new binding site, maintains the full potency of killing found in the native toxin but with the high cell-type specificity of A chain conjugates.[9,12]

Cell lines derived from medulloblastoma, glioblastoma, and breast carcinoma were chosen to assess the *in vitro* efficacy of CRM 107-based immunotoxins for treatment of tumors of the CSF compartment. These three types of tumors were selected because they represent examples of CNS tumors which are often difficult to treat with conventional forms of therapy and, in the case of breast carcinoma and medulloblastoma, frequently cause morbidity and mortality from leptomeningeal involvement. These *in vitro* results indicate that immunotoxin therapy offers certain advantages over existing treatments for these tumors.

Medulloblastoma comprises 20% of all brain tumors in children.[3] Because of this tumor's high incidence of recurrence and propensity for dissemination through the CSF, these children require postoperative radiation therapy which usually impairs their intellectual and physical development.[3,21] A more targeted treatment

such as immunotoxin therapy would be especially useful for this type of tumor. Intrathecal immunotoxin treatment of medulloblastoma has the added advantage of free access to the tumor cells within the CSF, avoiding the potential problem of limited penetration into the tumor mass.

Glioblastoma, the most common primary malignant brain tumor, is rapidly fatal. The best treatment currently available (surgery, radiation therapy, and systemic chemotherapy) results in median survival times of less than 1 year.[27,28] Inasmuch as most treatment failures occur because of local recurrence of glioma,[10] immunotoxins administered directly into the CSF or tumor to avoid delivery problems caused by the blood-brain barrier may provide additional tumor response and enhance log kill required for effective treatment of glioblastoma.

A variety of carcinomas, most commonly those involving the breast, metastasize to the leptomeninges. The incidence of meningeal carcinomatosis in breast cancer is believed to be rising due to improvements in the management of systemic disease.[30] Diffuse secondary involvement of the leptomeninges without focal parenchymal involvement is occasionally reported.[26,30] These patients would be particularly suited for immunotoxin therapy since the problems involved with penetration of a solid tumor mass by immunotoxins would be minimal.

This study compared the efficacy of immunotoxins made with CRM 107 and RTA on representative cell lines derived from medulloblastoma, glioblastoma, and breast carcinoma. A monoclonal antibody to the TfR, 454A12, was linked to each toxin and the *in vitro* toxicity was examined. Using a 24-hour inhibition of protein synthesis assay, both immunotoxins killed these cell lines at concentrations ranging between 10^{-10} and 10^{-11} M. However, it was found that CRM 107 immunotoxins kill at a much faster rate than the RTA immunotoxins. A shorter incubation time (3 hours) for the *in vitro* assay demonstrates these kinetic differences. In these assays, 454A12-CRM 107 is 10- to 1000-fold more toxic than 454A12-RTA. The CRM 107 conjugates displayed steep dose-response curves, again indicative of rapid killing. This increase in the rate of killing by CRM 107 immunotoxins compared to those made with RTA probably reflects the potentiating effect derived from the B chain entry function.

It is logical that an immunotoxin that kills at a faster rate will ultimately produce greater log kill than a slower-acting immunotoxin. Laurent, *et al.*,[15] demonstrated this principle using a clonogenic assay. They found that immunotoxins with the fastest kinetics of killing also exhibited the greatest log kill of human lymphocytes. Our results predict that the *in vivo* efficacy of CRM 107 immunotoxins may surpass that of RTA immunotoxins.

CRM 107 immunotoxins offer another advantage over RTA immunotoxins. Most individuals are immunized against DT, as evidenced here by high titers of circulating neutralizing antibody. Therefore, any "leakage" of the CRM 107 immunotoxin from the CSF into the systemic vasculature would be quickly neutralized by circulating anti-DT immunoglobulin.

Unlike many monoclonal antibodies, Tf cross-reacts among species.[1] Therefore, by linking Tf with CRM 107 we were able to evaluate the toxicity of the conjugate administered intrathecally in both guinea pigs and rhesus monkeys. Problems encountered in the periphery which would limit the efficacy of Tf-CRM 107 (that is, high levels of Tf and circulating anti-DT antibodies) do not appear to occur in the CSF. In rhesus monkeys, the highest dose of Tf-CRM 107 tested (2×10^{-9} M) was without toxicity. Continuing toxicity trials to test higher concentrations will determine the maximum tolerated dose. In guinea pigs, the maximum tolerated dose in the CSF was 2×10^{-9} M. The concentration of Tf-CRM 107 required to kill 50% of the cells derived from medulloblastoma, glioblastoma, or breast carcinoma *in vitro* ranged from 1×10^{-10} M to 4×10^{-13} M. We were therefore able to achieve a concentration of the conjugate in the CSF that was from 20- to 5000-fold higher than the level effective *in vitro* without detectable animal toxicity.

CRM 107 conjugates represent a significant advance in immunotoxin efficacy. They combine a high degree of tumor specificity, the ultimate degree of potency (1 molecule/cell is sufficient to kill), with extremely rapid killing to produce a therapeutic window of up to 5000-fold. These factors, together with the advantages offered by compartmentalized treatment, demonstrate that CRM 107 immunotoxins have considerable potential for the treatment of leptomeningeal neoplasia.

Acknowledgments

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Address reprint requests to: Virginia G. Johnson, Ph.D., Surgical Neurology Branch, NINCDS, National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892.

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1. Cancer Treatment and Research, 1988, Vol. 37, pp. 113-122
2. J of Neurosurgery, 1989 Feb, 70(2):240-248
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8. Genetic engineering of immunotoxins

R.J. Youle, L. Greenfield, and V.G. Johnson

Introduction

Genetic engineering offers many advantages for the 1) production of immunotoxins (ITs) and 2) design of more effective reagents. In Chapter 9, J.R. Murphy discusses the use of genetic engineering to link genes together resulting in chimeric proteins. This review covers the first applications of genetic engineering technology to improve ITs by studying and altering toxin B chains and broadening the therapeutic index between target and nontarget cells.

Ricin

Ricin is an extremely potent toxin isolated from castor bean seeds [1]. Ricin is synthesized during seed development and packaged into protein bodies within endosperm cells [2, 3]. The ricin gene has been cloned into *E. coli* by several groups [4, 5], and the nucleotide sequence closely corresponds with the protein sequence [6, 7]. The cDNA and genomic sequences show that ricin has a leader sequence that presumably initiates contranslational compartmentalization of ricin into the endoplasmic reticulum and that the two polypeptide chains, A and B, are initially synthesized as one polypeptide precursor joined by 12 amino acids [4, 5]. The precursor is proteolytically cleaved to yield two disulfide-linked subunits within the seed [8]. The toxin is post-translationally glycosylated on both A and B chains with high mannose branched chains containing some xylose and fucose [9, 10].

Two isomers of ricin, called ricin D and E, have only 12 amino acid differences in sequence [11] and differ in the structure of the binding site [12]. Therefore, the differences in sequence between ricins D and E indicate amino acids and domains involved in the galactose binding site. The variation of ricin sequence between varieties and species of castor bean is unknown; different investigators use different strains of seed. Halling et al. [4] showed that the ricin sequence is one of a family closely related genes.

Frankel, A.E., (ed.), Immunotoxins.

A hemagglutinin closely related to ricin also exists in the protein bodies of castor bean seeds [13]. This protein is homologous to ricin and has been cloned and sequenced [14]. The agglutinin is 120,000 kilodaltons and is comprised of two A chains and two B chains. The ricin E, B chain appears to have arisen from a recombination of the ricin D, B chain and the agglutinin B chain [14]. The function of ricin and the agglutinin in the plant is unknown.

Production

M. Piatak and colleagues at Cetus have obtained high yield expression of ricin A chain in *E. coli* [15]. The recombinant ricin A chain (rRTA) is absolutely free of ricin B chain and has no carbohydrate residues covalently attached. The LD_{50} of rRTA in mice is 25 mg/kg, compared to .003 mg/kg of native ricin. The concentration of rRTA needed to inhibit protein synthesis in cell-free translation assays is 3 ng/ml, which compares favorably with the native ricin A chain activity [1]. The recombinant ricin A chain has been crystallized [16].

Recombinant ricin A chain has been linked to monoclonal antibodies and assayed for toxicity in vitro and in vivo. A monoclonal antibody specific for the human transferrin receptor (454A12) was linked to ricin A chain and recombinant ricin A chain [17]. Both ITs had the same potency to target cells in vivo and in vitro. In a nude mouse model of ovarian cancer, the rRTA IT could more than double the length of survival of intraperitoneal tumor-bearing animals.

In another study, recombinant ricin A ITs were compared to intact ricin ITs [18]. Cloned ricin A chain was linked to a monoclonal antibody (M6) specific for the idiotype domain on L_2C guinea pig leukemia cells. In vitro, the conjugate (M6-rRTA) inhibited protein synthesis of L_2C cells 40% at 5×10^{-5} M after five hours. The toxicity was potentiated by monensin. The same monoclonal antibody (M6) linked to ricin (M6-ricin) inhibited protein synthesis 98% under the same assay conditions. The intact ricin conjugate was more potent in vitro than the ricin A chain conjugate. For tumor therapy in vivo, the cloned ricin A chain conjugate was compared with monoclonal antibody alone and with intact ricin conjugate. Table 1 shows that a hierarchy in potency was found. M6 antibody was significantly better than no treatment and killed about 90% of the tumor cells in vivo at 3000 μg/kg. M6-rRTA killed about 90% of the cells at a 100-fold lower concentration than the M6 antibody alone, 30 μg/kg. At 3000 μg/kg M6-rRTA was more effective than an equivalent dose of antibody, killing up to 5 logs of 99.999% of the tumor cells. This shows that the recombinant ricin A chain conjugate was extremely effective in vivo and more potent than antibody alone.

Table 1[a]. Tumor therapy o

Treatment
Buffer
M6 Antibody
3000 μg/kg
M6-rRTA
35 μg/kg
M6-rRTA
3500 μ/kg
M6-Ricin
30 μg/kg

[a] Adapted from Gregg et a
[b] Calculated based on a 18-

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compared to intact ricin
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t 3000 μg/kg M6-rRTA
›ody, killing up to 5 logs
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Table 1[a]. Tumor therapy of guinea pigs injected with L_2C leukemia cells

Treatment	*Mean Survival Time*	*Log Kill of Tumor Cells*[b]
Buffer	15	0
M6 Antibody 3000 μg/kg	18	1
M6-rRTA 35 μg/kg	18	1
M6-rRTA 3500 μ/kg	34	5
M6-Ricin 30 μg/kg	31	5

[a] Adapted from Gregg et al., 1987
[b] Calculated based on a 18-hour doubling time of L_2C cells

Specificity or therapeutic index

Ricin B chain can potentiate ricin A chain ITs [19–21]. Furthermore, intact ricin ITs are often more potent than ricin A chain ITs [22]. Gregg and colleagues [18] compared the activity of an rRTA IT with that of an intact ricin IT in vivo. Table 1 shows that the intact ricin IT killed 5 logs of tumor cells at a dose 100-fold lower than the rRTA IT. The B chain of ricin can therefore potentiate activity in vivo. Since both the rRTA and intact ricin ITs were used at doses within 3–5-fold of lethal levels and effected the same tumor cell kill, the conclusion was made that, though the ricin IT was 100 times more potent, both reagents had the same therapeutic index.

The ricin and diphtheria toxin B subunits have two known functions: 1) binding cell surface receptors, and 2) facilitating entry of the toxin. The binding activity of the B chains causes nontarget cell toxicity of ITs and the entry activity increases the toxicity or potency of ITs to target cells. One way to increase the therapeutic index of ITs would be to block the binding activity and maintain the entry activity of the B chain. Recent work indicates that this may not be possible for ricin. Chemical inactivation of the galactose binding site on ricin was shown to block the toxicity of ITs [23], though Vitetta [24], using a different chemical modification of ricin and including NH_4+Cl^- in the assays, came to the opposite conclusion. Goldmacher [25] found that cells lines selected for resistance to an intact ricin IT were resistant to ricin and had a decreased level of ricin receptors. This result indicates that ricin receptors are needed for the antibody-mediated toxicity of the IT. In addition, hybridoma cells that contain intracellular monoclonal antibody that specifically blocked ricin binding, blocked ricin toxicity from within cells [26]. A model consistent with these results is that ricin uses the galactose binding activity on the B chain within cells, possibly within the Golgi apparatus, to enter the cytosol. According to this model, blockage of the galactose-binding

activity on intact ricin ITs would block the entry to the cytosol. Therefore, site-specific mutagenesis that blocks the nontarget cell killing of ricin would not increase the therapeutic index of ITs. Reversible modification of the galactose-binding site that blocked binding outside target cells and exposed binding within cells may, however, generate reagents with improved therapeutic indices. Recent work with diphtheria toxin discussed later, in contrast to ricin, shows separation of binding and entry is possible, and such modified diphtheria toxin conjugates have a greater therapeutic index.

Stability

The reticuloendothelial system has at least two clearance mechanisms specific for carbohydrates [27]. Hepatocytes have receptors that bind and internalize carbohydrates and glycoproteins containing terminal galactose, and Kupfer cells have receptors that bind and internalize carbohydrates and glycoproteins containing terminal mannose residues [28, 29]. Native ricin has complex carbohydrate groups that have terminal mannose on both the A and B subunits [9], which result in specific binding and internalization of ricin by Kupfer cells. This causes rapid clearance of ricin and ricin A chain containing ITs in vivo. Efforts have been made to remove the carbohydrate from ricin A chain to prevent the rapid destruction of ITs in vivo [10]. Recombinant ricin A chain lacks carbohydrate and will not be cleared by the mannose-specific uptake pathway of the reticuloendothelial system as rapidly as native ricin A chain.

Recombinant ricin A chain has a number of advantages over native ricin A chain: 1) The production of clinically useful amounts is simplified, 2) the chance of contamination with ricin B chain is eliminated, and 3) the carbohydrate that causes rapid clearance of native ricin A chain is absent. Genetic engineering may also be used to modify toxin B chains and improve IT selectivity, as recently shown for diphtheria toxin [30, 31]. Such improvements in selectivity may or may not be accomplished for ricin or other toxins.

Diphtheria toxin

Diphtheria toxin, after proteolytic nicking, consists of two disulfide linked polypeptide chains. The A chain enzymatically inactivates EF-2 by ADP-ribosylation and blocks protein synthesis. The B chain has two activities: 1) binding cell surface receptors, and 2) translocation of the A subunit to the cytosol compartment. The mechanism of diphtheria toxin (DT) entry into cells is more clearly understood than the mechanism of ricin entry and appears to be different. Diphtheria toxin B subunit has four extremely hydrophobic regions that are capable of binding lipid [32, 33]. Upon exposure to pH below 5.5, the toxin undergoes a conformational change that exposes

A c

Diphtheria Toxin

CRM 45

MspSA

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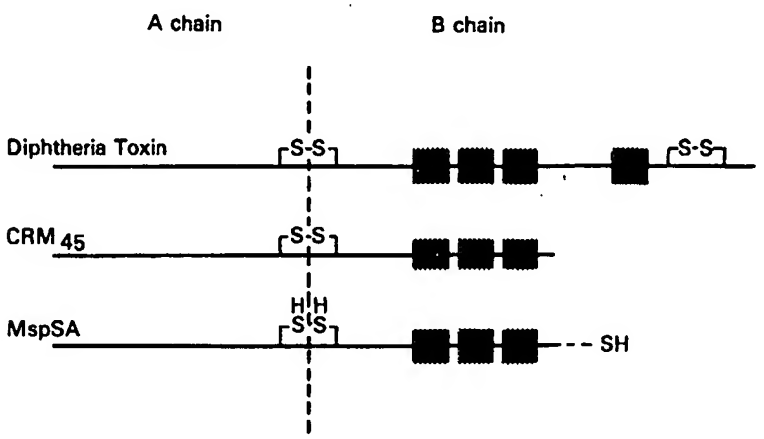

Figure 1. Domains of diphtheria toxin and fragments. Hydrophobic domains are marked by black boxes. Adapted from Colombatti, et al., 1986.

hydrophobic domains [34] and promotes toxin insertion into lipid bilayers [35]. That these events are important in toxin entry into cells is indicated by the fact that amines [36] or ionophores that increase the pH of intracellular vesicles block DT toxicity. Further, pulsing cells at low pH can mediate DT toxicity, even in the presence of ionophores [37, 38]. The hydrophobic regions of DT thought to be involved in membrane transport are shown on Figure 1 [32].

The receptor for DT on certain cells, such as the monkey kidney cell line Vero, appears to be the anion antiporter [39]. Polyphosphates such as ATP and phytic acid block DT binding [40]. The binding site on DT is in the 8 kilodalton C-terminal fragment of the B chain [41] that is missing in CRM45 (Figure 1). The serine at amino acid 508 is involved in DT binding and may lie in the binding site [31].

Greenfield and colleagues at Cetus cloned the fragment of DT corresponding to the CRM45 mutant into *E. coli* extending from amino acid 1 to 382 (Figure 1). In addition, they designed a spacer arm and a crosslinking site at the C-terminus. The nucleotide sequence corresponding to leu-pro-gly-thr-gly-ser-gly-pro$_6$-ser-gly-ser-gly-thr-cys was synthesized by solid-phase methodology and inserted adjacent to the C-terminus of the Msp restriction site at amino acid number 382. The new protein, called MspSA, was expressed in high yield and purified.

Specificity or therapeutic index

Colombatti and coworkers [30] linked MspSA to a monoclonal antibody specific for the T3 antigen on human T cells and compare its bioactivity to intact

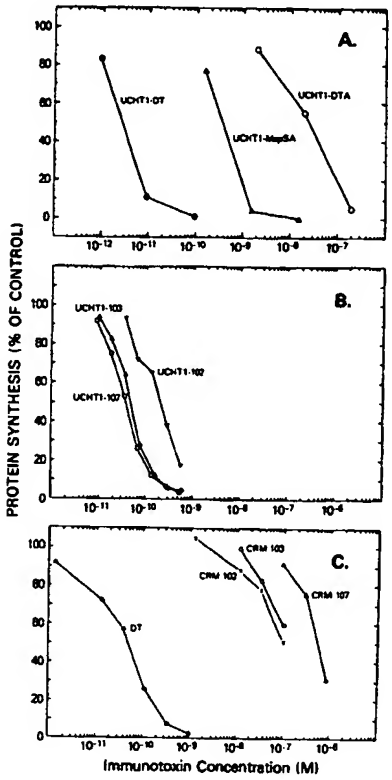

Figure 2. Potency of genctically engineered toxins and immunotoxins. A: Comparison of toxicity of a monoclonal antibody against human T cells (UCHT1) linked to DT A chain (○——○) or intact DT (■——■) or a genetically engineered fragment to DT, called MspSA (△——△), against the human T cell line Jurkat. (Adapted from *J. Biol. Chem.* 261: 3030–3035.) B: Immunotoxins made with diphtheria toxin mutants with point mutations blocking toxin binding assayed for toxicity to Jurkat cells. C: Toxicity of diphtheria toxin and mutants with point mutations blocking bindings assayed on Jurkat cells. Adapted from *Science*.

DT and A chain ITs. The diphtheria toxin A chain IT was 10,000-fold less potent than the intact DT IT (Figure 2A). The MspSA IT, which contained half of the native DT, was intermediate in potency between the A chain and intact toxin reagents (Figure 2A). The N-terminal 17 kilodalton region of DTB which was contained in the MspSA, therefore, potentiated IT activity 100-fold. Since the C-terminal region of DT contains the cell-surface binding site, this 100-fold activation appears to be due to entry activity expressed by MspSA and not expressed by DT A chain. However, the intact DT was 100-fold more potent than the recombinant MspSA IT. Since the MspSA IT lacks the C-terminal binding site as well as one hydrophobic domain, it was

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rophobic domain, it was

not possible to tell whether this 100-fold potentiation was distinct from binding or not. The most elegant way to separate binding and entry would be to use point mutagenesis to block the B chain binding site.

We recently identified point mutations in DT that block binding and toxicity [31]. Three point mutants of DT have toxicity decreased 1000- to 10,000-fold (Figure 2C) and have binding activity decreased 100- to 9000-fold. Sequence analysis of the mutants identifies several amino acids involved in binding, including serine at position 508 and either leucine at position 325 or serine at position 525.

Upon linking the inactivated mutants CRM107 and CRM103 to a monoclonal antibody, full toxicity was restored, indistinguishable from the native DT linked to the same antibody (Figure 2B). This shows that the only defect in CRM107 that reduced toxicity 10,000-fold could be fully reversed by adding a new binding domain. This demonstrates that the binding function is not required for the entry activity of the B chain. The selectivity of the new reagent is increased 10,000-fold over the 100-fold selectively of the native DT IT, yielding a 2×10^5-fold selective toxicity. This new reagent has the high potency of intact toxin ITs to target cells combined with the low toxicity of A chain ITs to nontarget cells. This genetically engineered IT has three separable domains used to mediate cell-type-specific toxicity: 1) a monoclonal antibody binding domain, 2) the B subunit with full translocation activity, and 3) the A chain with enzymatic activity to block protein synthesis.

Stability

Use of intact toxins genetically altered in their binding sites offers many advantages over the use of A chain ITs. First, as described, the therapeutic index can be orders of magnitude greater than for A chain ITs. In addition, a more stable linkage between intact toxin and antibody can be used than between the A chain and antibody. The A chain must be separated from the B in the cytosol for maximal enzymatic inhibition of protein synthesis [42, 43]. Therefore, A chain ITs linked to monoclonal antibodies by disulfide bonds are more effective than those made with more stable thioether bonds [44]. However, the exogenous disulfide bond between A chains and antibody can be readily broken in the blood [45]. In contrast, intact toxins can be linked to antibodies by nonreducible thioether bonds because the A chain is still linked to the complex by the disulfide bond to the B chain, allowing efficient inhibition of protein synthesis [46, 47], and the endogeneous disulfide bond is stabilized in serum by the association of the A and B chains [48].

A chains have also been shown to be more susceptible to proteolysis than A chains associated with B chains [6]. In vivo stability to proteolysis of intact toxin ITs may be greater than that of A chain ITs.

Conclusion

ITs show promise as a new class of chemotherapeutic reagents. They can be extremely tumor selective in vitro and in vivo in certain body compartments. However, they currently have many limitations that must be overcome to optimize their in vivo efficacy. Genetic engineering has already been used to improve IT production, stability, and selectivity. New ITs engineered at the gene level to overcome the current limitations will undoubtedly have greatly improved pharmacologic activities and clinical value.

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9. Diphtheria-r
A molecular
developmen

John R. Murphy

Introducti n

Over the past 15 year
understanding of the
molecule (see Chapte
tion that diphtherial
from an ordered serie
cell. For example, the
the following steps: 1)
2) internalization of
acidification of the en
which leads to 4) the
into the cytosol, and
dependent adenosine
tion factor 2. Modifie
location of polypeptid
('P') site on the ribos
series of steps results
intact sensitive eukary

The analysis of p
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theria toxin (58,348 M
mild trypsin digestion
fragment A (21,167 M
chemical analysis of n
to diphtheria toxin in
contained entirely wit
binding domain of the
15,000 dalton region
the structural gene tha
or the receptor bindin
for intact cells.

Frankel, A.E., (ed.). Immuno

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     (FILE 'HOME' ENTERED AT 14:52:11 ON 13 FEB 2003)

     FILE 'MEDLINE, BIOSIS, CANCERLIT, LIFESCI, BIOTECHDS, CAPLUS' ENTERED AT
     14:53:56 ON 13 FEB 2003
L1        11029 S DIPHTHERIA(W)TOXIN#
L2          189 S DIPTHERIA(W)TOXIN#
L3        13671 S (UROKINASE OR URINARY)(W)PLASMINOGEN
L4        11096 S L1 OR L2
L5           10 S L4 AND L3
L6            8 S L5 AND PY<2002
L7            4 DUP REM L6 (4 DUPLICATES REMOVED)

     FILE 'PCTFULL, USPAT2, EUROPATFULL' ENTERED AT 15:02:00 ON 13 FEB 2003

=> s (DIPHTHERIA(W)TOXIN# or DIPTHERIA(W)TOXIN#)
L8        3764 (DIPHTHERIA(W) TOXIN# OR DIPTHERIA(W) TOXIN#)

=> s ((UROKINASE OR URINARY)(W)PLASMINOGEN) or uPa
L9        1556 ((UROKINASE OR URINARY)(W) PLASMINOGEN) OR UPA

=> s l8(s)l9
L10          4 L8(S) L9
```

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     (FILE 'HOME' ENTERED AT 19:30:47 ON 13 FEB 2003)

     FILE 'MEDLINE, BIOSIS, CANCERLIT, LIFESCI, BIOTECHDS, CAPLUS' ENTERED AT
     19:31:09 ON 13 FEB 2003
L1           972 S VALLERA?/AU
L2            71 S L1 AND DIPHTHERIA
L3             6 S L1 AND (UROKINASE(2W)PLASMINOGEN OR UPA)
L4             6 S L3 AND L2
L5             0 S L4 AND PY<2002
L6             2 DUP REM L4 (4 DUPLICATES REMOVED)
L7           427 S (HALL,W#)/AU OR (HALL W#)/AU OR (HALL, W#)/AU

=> s l7 and (urokinase(2w)plasminogen or upa)
L8             0 L7 AND (UROKINASE(2W) PLASMINOGEN OR UPA)

=> s l7 and diphtheria
L9             0 L7 AND DIPHTHERIA
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     (FILE 'HOME' ENTERED AT 10:17:16 ON 14 FEB 2003)

     FILE 'MEDLINE, BIOSIS, CANCERLIT, LIFESCI, CAPLUS, BIOTECHDS' ENTERED AT
     10:17:36 ON 14 FEB 2003
L1          8396 S COLICIN
L2            69 S D-ENDOTOXIN
L3          4277 S DELTA(A)ENDOTOXIN
L4          1225 S ANTHRAX(A)TOXIN
L5          8450 S TETANUS(A)TOXIN
L6           180 S (BOTULISM OR BOLTULINUM)(A)TOXIN
L7          3380 S PSEUDOMON?(W)(EXOTOXIN)
L8            69 S (INTERNALI?)(W)DOMAIN#
L9             0 S L8 AND L1
L10            0 S L2 AND L8
L11            0 S L3 AND L8
L12            0 S L4 AND L8
L13            0 S L5 AND L8
L14            0 S L6 AND L8
L15            1 S L7 AND L8

=> s (internali? or transloca?)(3a)domain#
L16        2555 (INTERNALI? OR TRANSLOCA?)(3A) DOMAIN#

=> s l1 and l16
L17           87 L1 AND L16

=> s l2 and l16
L18            0 L2 AND L16

=> s l3 and l16
L19            1 L3 AND L16

=> s l4 and l16
L20           14 L4 AND L16

=> s l5 and l16
L21           18 L5 AND L16

=> s l6 and l16
L22            0 L6 AND L16

=> s l17/ti
L23           20 L17/TI

=> s l23 or l19 or l20 or l21
L24           53 L23 OR L19 OR L20 OR L21

=> dup rem l24
PROCESSING COMPLETED FOR L24
L25           25 DUP REM L24 (28 DUPLICATES REMOVED)
```

```
=> s diphtheria
L1       33091 DIPHTHERIA

=> s lrp or (ldl(2w)receptor) or ((a2 or alpha2)(w)macroglobulin) or
a2macroglobulin# or (alpha(w)2(w)macroglobulin#)
   6 FILES SEARCHED...
L2       44327 LRP OR (LDL(2W) RECEPTOR) OR ((A2 OR ALPHA2)(W) MACROGLOBULIN)
               OR A2MACROGLOBULIN# OR (ALPHA(W) 2(W) MACROGLOBULIN#)

=> s l1 and l2
L3          56 L1 AND L2

=> s l3 and py<2002
   2 FILES SEARCHED...
   4 FILES SEARCHED...
L4          52 L3 AND PY<2002

=> dup rem l4
PROCESSING COMPLETED FOR L4
L5           29 DUP REM L4 (23 DUPLICATES REMOVED)

=> d ibib abs tot
```

1: CAA25302. diphtheria toxin ...[gi:758186]

BLink, Domains, Links

```
LOCUS       CAA25302                 560 aa            linear   PHG 30-MAR-1995
DEFINITION  diphtheria toxin [Corynebacterium diphtheriae virus].
ACCESSION   CAA25302
VERSION     CAA25302.1  GI:758186
DBSOURCE    embl locus NCCDTOX, accession X00703.1
KEYWORDS    .
SOURCE      Corynebacterium diphtheriae virus
  ORGANISM  Corynebacterium diphtheriae virus
            Viruses.
REFERENCE   1  (residues 1 to 560)
  AUTHORS   Collier,R.J. and Kaplan,D.A.
  TITLE     Immunotoxins
  JOURNAL   Sci. Am. 251, 44-52 (1984)
COMMENT     The hydrophobic region of the B chain is responsible for the
            toxin's insertion into the endosomal membrane and therefore for the
            A chain's emergence from an endosome into the cytoplasm. The
            disulfide bond (S-S) between Cys(186) and Cys(201) links the the
            two chains.
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:28368"
     Protein         1..560
                     /product="diphtheria toxin"
     sig_peptide     1..25
                     /note="signal peptide"
     CDS             1..560
                     /coded_by="X00703.1:1..1683"
                     /transl_table=11
                     /db_xref="SWISS-PROT:P00588"
ORIGIN
        1 msrklfasil igallgigap psahagaddv vdssksfvme nfssyhgtkp gyvdsiqkgi
       61 qkpksgtqgn ydddwkgfys tdnkydaagy svdnenplsg kaggvvkvty pgltkvlalk
      121 vdnaetikke lglslteplm eqvgteefik rfgdgasrvv lslpfaegss sveyinnweq
      181 akalsvelei nfetrgkrgq damyeymaqa cagnrvrrsv gsslscinld wdvirdktkt
      241 kieslkehgp iknkmsespn ktvseekakq yleefhqtal ehpelselkt vtgtnpvfag
      301 anyaawavnv aqvidsetad nlekttaals ilpgigsvmg iadgavhhnt eeivaqsial
      361 sslmvaqaip lvgelvdigf aaynfvesii nlfqvvhnsy nrpayspghk tqpflhdgya
      421 vswntvedsi irtgfqgesg hdikitaent plpiagvllp tipgkldvnk skthisvngr
      481 kirmrcraid gdvtfcrpks pvyvgngvha nlhvafhrss sekihsneis sdsigvlgyq
      541 ktvdhtkvns klslffeiks
```